

-39-

CLAIMS

1. A method for preventing the toxic effects of a superantigen by treatment with a molecule, wherein said molecule elicits antibody production without inducing T cell activation.
5
2. The method of claim 1 wherein said molecule is a mutated superantigen.
10
3. The method of claim 1 wherein said molecule is a modified superantigen.
15
4. A molecule comprising a mutated superantigen.
15
5. A molecule comprising a modified superantigen.
20
6. A method of modifying T cell response elicited by an antigen comprising administering a molecule which interacts with specific V β elements of T cell receptors (TCR).
25
7. The method of claim 6 wherein said molecule is a mutated superantigen.
25
8. The method of claim 6 wherein said molecule is a modified superantigen.
30
9. A method for treating the toxic effects of superantigen by treatment with a molecule, wherein said molecule elicits antibody production without inducing T cell activation.
30

19.07.94 7
LUCAS & CO.

Chartered Patent Agents
European Patent Attorneys

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ENGLAND

DOO
Telephones: 0883-626211

15th July 1994

The European Patent Office
PB5818 Patentlaan 2
NL-2280 HV Rijswijk
Netherlands

033295.7

Dear Sirs

re: New European Patent Application
Based on PCT Patent Applctn No. PCT/US93/00839
(Publication No. WO 93/14634) ✓
(Filed 28 Jan 93)
National Jewish Center for Immunology and ✓
Respiratory Medicine
"Protective Effects of Mutated Superantigens"
Case: NJH223.1/EP ✓

This is simply to advise you that we have been instructed to act on behalf of National Jewish Center for Immunology and Respiratory Medicine in connection with the prosecution of the European Regional Phase of this application.

Our Clients have instructed us that they wish to proceed in all of the designated states, which are: Austria, Belgium, Switzerland (including Liechtenstein), Germany, Denmark, Spain, France, UK, Greece, Ireland, Italy, Luxembourg, Monaco, Holland, Portugal and Sweden.

We have today sent a copy of this letter to your Cash & Accounts department in Munich, together with a cheque covering:

1. National Fee;
2. Designation Fees (16)
3. Additional Claim Fees (0);
4. Search fee (80%); and
5. Substantive Examination Fee (100%).

19.07.94

1994

Please note that we have only paid 80% of the Search Fee as the International Search was carried out by the US Searching Authority. Furthermore, we have paid 100% of the Substantive Examination fee as International Preliminary Examination was carried out by the US Patent Office.

Cont'd. . .

Would you please accept this letter as our formal request for Substantive Examination of this application.

Finally, would you please return the attached copy of this letter in the envelope provided to acknowledge safe receipt hereof.

Yours sincerely



Brian Lucas
Chartered Patent Agent
European Patent Attorney

FINANZ

GENERATED CODING

DOSSIER NR: 93904757.7

NR: US9300839

2 939047577

LUCAS, BRIAN RONALD ESQ LUCAS & CO

| | | | | |
|---------|---------|---|----------|---------|
| 002 GBP | 608,00 | ✓ | SFEE11 | 150794! |
| 005 GBP | 2240,00 | ✓ | DEST13AT | 150794! |
| | | | DEST13BE | 150794! |
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M. PEIS

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Europäisches
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135 Westhall Road
Warlingham
Surrey CR6 9HJ
GRANDE BRETAGNE

Datum/Date

26/10/94

| | | | |
|----------------------------------------------------------------------------------------------------------------------------|--------------------------------------------------------------------|-----------------|-----------|
| Zeichen/Ref./Réf. | Anmeldung Nr./Application No./Demande n°./Patent Nr No./Brevet n°. | | |
| NJH223.1/EP | PCT/US9300839 | 93904757.7-2110 | / 0626805 |
| Anmelder/Applicant/Demandeur//Patentinhaber/Propriétaire NATIONAL JEWISH CENTER FOR IMMUNOLOGY AND RESPIRATORY MEDICINE | | | |

**NOTIFICATION OF EUROPEAN PUBLICATION NUMBER AND INFORMATION
ON THE APPLICATION OF ARTICLE 67(3) EPC**

The provisional protection under Article 67(1) and (2) EPC in the individual Contracting States becomes effective only when the conditions referred to in Article 67(3) EPC have been fulfilled (for further details: see information brochure of the European Patent Office "National Law relating to the EPC" and additional information in the Official Journal of the European Patent Office).

Pursuant to Article 158(1) EPC the publication under Article 21 PCT of an international application for which the European Patent Office is a designated Office takes the place of the publication of a European patent application.

The bibliographic data of the above-mentioned EURO-PCT application will be published on 07.12.94 in Section I.1 of the European Patent Bulletin.

The European publication number is 0626805.

In all future communications to the European Patent Office, please quote the application number plus Directorate number.

RECEIVING SECTION





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Lucas & Co.
135 Westhall Road
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Surrey CR6 9HJ
GRANDE BRETAGNE

COPY

Datum/Date 24. 10. 95

| | |
|-----------------------------------------------------------------------------------------------------------------------|------------------------------------------------------------------------------------------|
| Zeichen/Ref./Réf. NJH223.1/EP | Anmeldung Nr./Application No./Demande n°./Patent Nr./Patent No./Brevet n°. 93904757.7 |
| Anmelder/Applicant/Demandeur/Patentinhaber/Proprietor/Titulaire NATIONAL JEWISH CENTER FOR IMMUNOLOGY AND RESPIRAT | |

COMMUNICATION

The European Patent Office herewith transmits

- the European search report
- the declaration under Rule 45 EPC
- the partial European search report under Rule 45 EPC
- the supplementary European search report concerning the international application under Article 157(2) EPC relating to the above-mentioned European patent application. Copies of the documents cited in the search report are enclosed.

The following specifications given by the applicant have been approved by the Search Division :

Abstract

Title

Figure

The abstract was modified by the Search Division and the definitive text is attached to this communication.

The following figure will be published with the abstract, since the Search Division considers that it better characterises the invention than the one indicated by the applicant.

Figure:

Additional copy(copies) of the documents cited in the European search report.



24. 10. 1995
Marcos Bello



| DOCUMENTS CONSIDERED TO BE RELEVANT | | | | | |
|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|---------------------------------------------------------------|-----------------------------------------|----------------------|
| Category | Citation of document with indication, where appropriate, of relevant passages | Relevant to claim | CLASSIFICATION OF THE APPLICATION (Int.Cl.5) | | |
| X | JOURNAL OF IMMUNOLOGY, vol. 147, no. 10, 15 November 1991 BALTIMORE US, pages 3274-3281, GROSSMAN D. ET AL. * the whole document * --- | 1-9 | A61K37/00 A61K39/02 C07K13/00 C07K15/00 C12N15/00 | | |
| X | INFECTION AND IMMUNITY, vol. 58, no. 9, September 1990 WASHINGTON US, pages 3020-3028, BLANCO L. ET AL. * the whole document * --- | 1-9 | | | |
| D, X | SCIENCE, vol. 248, 11 May 1990 LANCASTER, PA US, pages 705-711, MARRACK P. ET AL. * the whole document * --- | 1-9 -/- | | | |
| <table border="1"> <tr> <td>TECHNICAL FIELDS SEARCHED (Int.Cl.5)</td> </tr> <tr> <td>C07K A61K C12N</td> </tr> </table> | | | | TECHNICAL FIELDS SEARCHED (Int.Cl.5) | C07K A61K C12N |
| TECHNICAL FIELDS SEARCHED (Int.Cl.5) | | | | | |
| C07K A61K C12N | | | | | |
| INCOMPLETE SEARCH | | | | | |
| <p>The Search Division considers that the present European patent application does not comply with the provisions of the European Patent Convention to such an extent that it is not possible to carry out a meaningful search into the state of the art on the basis of some of the claims</p> <p>Claims searched completely :</p> <p>Claims searched incompletely :</p> <p>Claims not searched :</p> <p>Reason for the limitation of the search:</p> <p>see sheet C</p> | | | | | |
| 1 | Place of search THE HAGUE | Date of completion of the search 5 October 1995 | Examiner Moreau, J | | |
| CATEGORY OF CITED DOCUMENTS | | <p>T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons & : member of the same patent family, corresponding document</p> | | | |
| <p>EPO FORM 1503 01.82 (P01C20)</p> | | | | | |



| DOCUMENTS CONSIDERED TO BE RELEVANT | | | CLASSIFICATION OF THE APPLICATION (Int.Cl.5) |
|-------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------|-------------------|----------------------------------------------|
| Category | Citation of document with indication, where appropriate, of relevant passages | Relevant to claim | |
| D, P, X | THE JOURNAL OF EXPERIMENTAL MEDICINE, vol. 175, February 1992 pages 387-396, KAPPLER J.W. ET AL. * the whole document * ---- | 1-9 | |
| A | WO-A-91 10680 (TERMAN D.) * page 53 - page 58 * ----- | 1-9 | |
| | | | TECHNICAL FIELDS SEARCHED (Int.Cl.5) |
| | | | |
| | | | |
| | | | |

ANNEX TO THE EUROPEAN SEARCH REPORT
ON EUROPEAN PATENT APPLICATION NO.

EP 93 90 4757

This annex lists the patent family members relating to the patent documents cited in the above-mentioned European search report.
The members are as contained in the European Patent Office EDP file on
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

05-10-1995

| Patent document cited in search report | Publication date | Patent family member(s) | Publication date |
|-------------------------------------------|---------------------|----------------------------|---------------------|
| WO-A-9110680 | 25-07-91 | EP-A- 0511306 | 04-11-92 |



EP 93 90 4757

-C-

Remark: Although claims 1-3 and 6-9 are directed to a method of treatment of the human/animal body (Art. 52(4) EPC) the search has been carried out and based on the alleged effects of the compound/composition



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GRANDE BRETAGNE



Datum/Date

27.10.95

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|-------------------------------------------------------------------------------------------------------------------------------------------|------------------------------------------------------------------------------------------------------------------------|
| Zeichen/Ref./Réf. NJH223.1/EP | Anmeldung Nr./Application No./Demande n° // Patent Nr./Patent No./Brevet n°. 93904757.7-2107 - PCT/US9300839 |
| Anmelder/Applicant/Demandeur//Patentinhaber/Proprietor/Titulaire NATIONAL JEWISH CENTER FOR IMMUNOLOGY AND RESPIRATORY MEDICINE | |

**PROCEEDING FURTHER WITH THE EUROPEAN PATENT APPLICATION PURSUANT TO
ARTICLE 96(1) AND RULE 51(1) EPC**

A supplementary European search report has been drawn up concerning
the above European patent application (publication no. 0626805).

Since you have filed a request for examination prior to the trans-
mission of the supplementary European search report, you are hereby
invited to indicate within

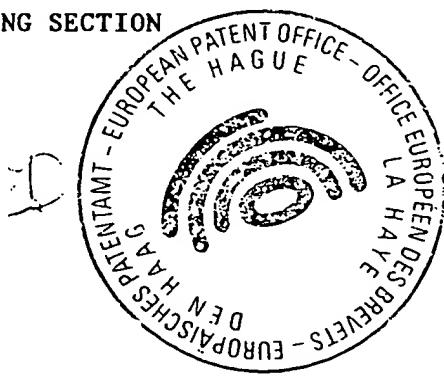
TWO MONTHS

of notification of this invitation whether you desire to proceed
further with the European patent application.

If you do not indicate in due time that you desire to proceed further
with the European patent application, it will be deemed to be withdrawn
(Art. 96(3) EPC).

If you wish you may comment on the supplementary European search report
and amend, where appropriate, the description, claims and drawings
(Rule 51(1) EPC).

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REGISTERED LETTER

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Chartered Patent Agents
European Patent Attorneys

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1st December 1995

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The European Patent Office
Erhardtstrasse 27
D-80298
Munich
Germany

LUCAS & CO.
Chartered Patent Agents
European Patent Attorneys

- 5 DEC 1995

135, Westhall Road, Warlingham,
Surrey, CR6 9HJ, England.

Dear Sirs

re: European Patent Application No 93 904757.7
(European Publication No. 0 626 805)
Based on PCT Patent Applctn No. PCT/US93/00839
(Publication No. WO 93/14634)
(Filed 28 Jan 93)
National Jewish Center for Immunology and
Respiratory Medicine
"Protective Effects of Mutated Superantigens"
Case: NJH223.1/EP

Following consideration of the Supplementary Search Report, we
confirm that this Application should be forwarded for Substan-
tive Examination.

Would you please return the attached copy of this letter to
acknowledge safe receipt hereof.

Yours faithfully


Brian
Brian Lucas/hb
Chartered Patent Agent
European Patent Attorney

Records:-

Status - 1 June 97

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| EPA EPO-OEB | | |
| DG 1 | | |
| Recu: | | |
| 06-12-1995 | | |
| 11 | | AM ZEPHAN |

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J.F. AGNES 07.12.1995

LUCAS & CO.

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European Patent Attorneys

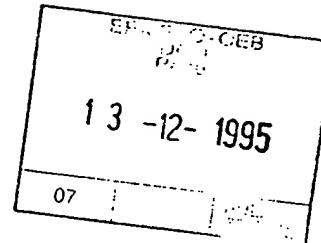
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Telephones: 01883-626211

1st December 1995

The European Patent Office
Erhardtstrasse 27
D-80298
Munich
Germany



Dear Sirs

2107
re: European Patent Application No 93 904757.7 5
(European Publication No. 0 626 805)
Based on PCT Patent Applctn No. PCT/US93/00839
(Publication No. WO 93/14634)
(Filed 28 Jan 93)
National Jewish Center for Immunology and
Respiratory Medicine
"Protective Effects of Mutated Superantigens"
Case: NJH223.1/EP

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confirm that this Application should be forwarded for Substan-
tive Examination.

Would you please return the attached copy of this letter to
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Yours faithfully


Brian Lucas/hb
Chartered Patent Agent
European Patent Attorney

Records:-

Status - 1 June 97

15
5 17. VI. 96 L

-39-

CLAIMS

1. A method for preventing the toxic effects of a superantigen by treatment with a molecule, wherein said molecule elicits antibody production without inducing T cell activation.
5
2. The method of claim 1 wherein said molecule is a mutated superantigen.
10
3. The method of claim 1 wherein said molecule is a modified superantigen.
15
4. A molecule comprising a mutated superantigen.
15
5. A molecule comprising a modified superantigen.
20
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25
7. The method of claim 6 wherein said molecule is a mutated superantigen.
30
8. The method of claim 6 wherein said molecule is a modified superantigen.
30
9. A method for treating the toxic effects of superantigen by treatment with a molecule, wherein said molecule elicits antibody production without inducing T cell activation.

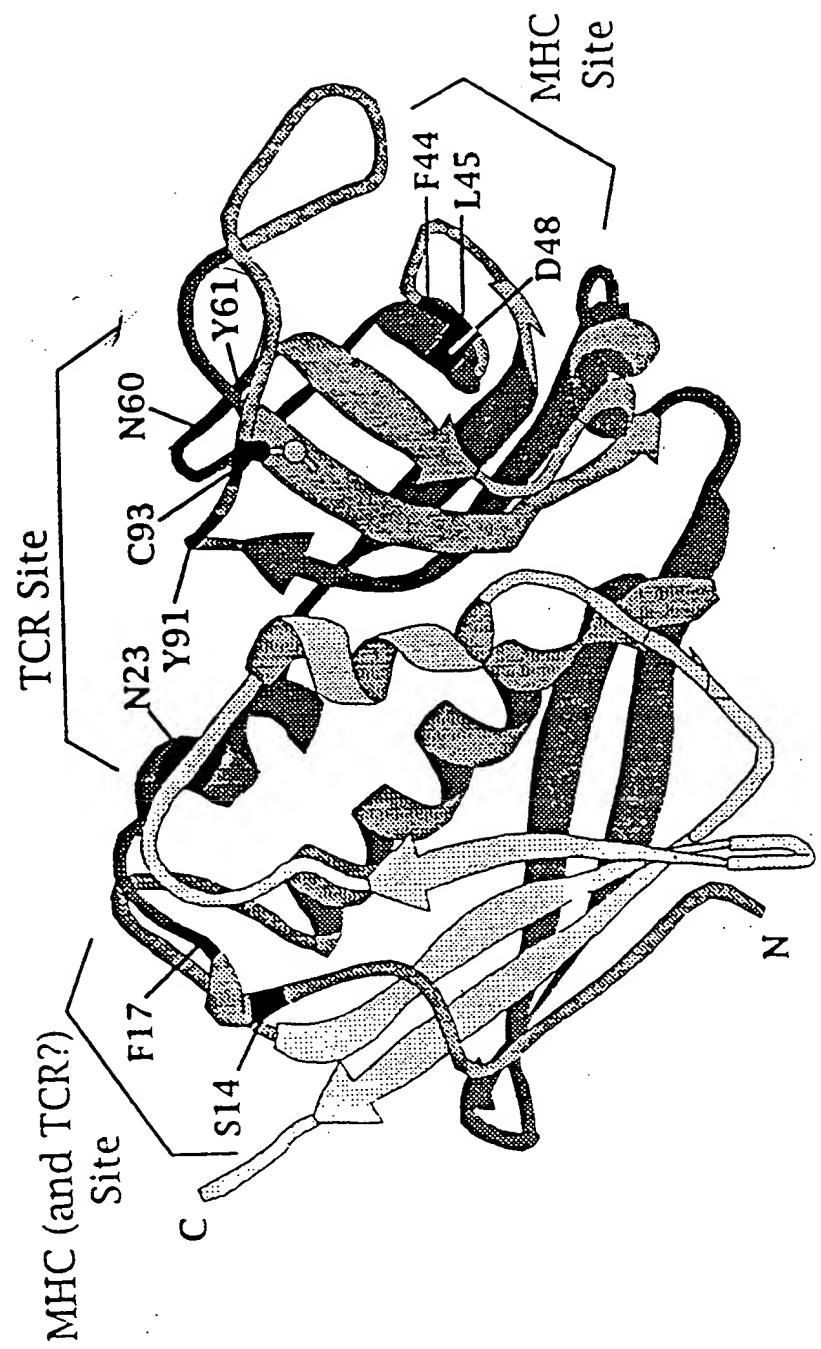


FIG. 1

8/12

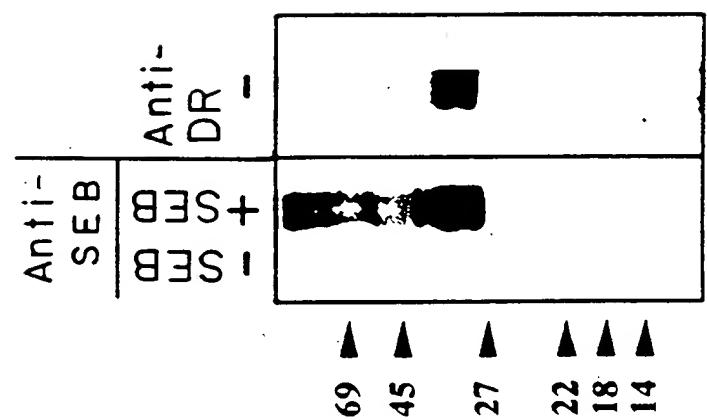


FIG. 2B

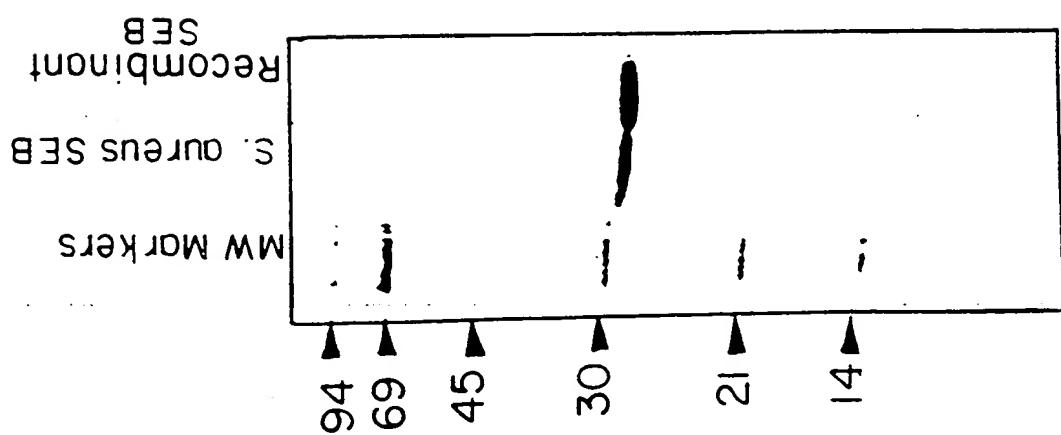
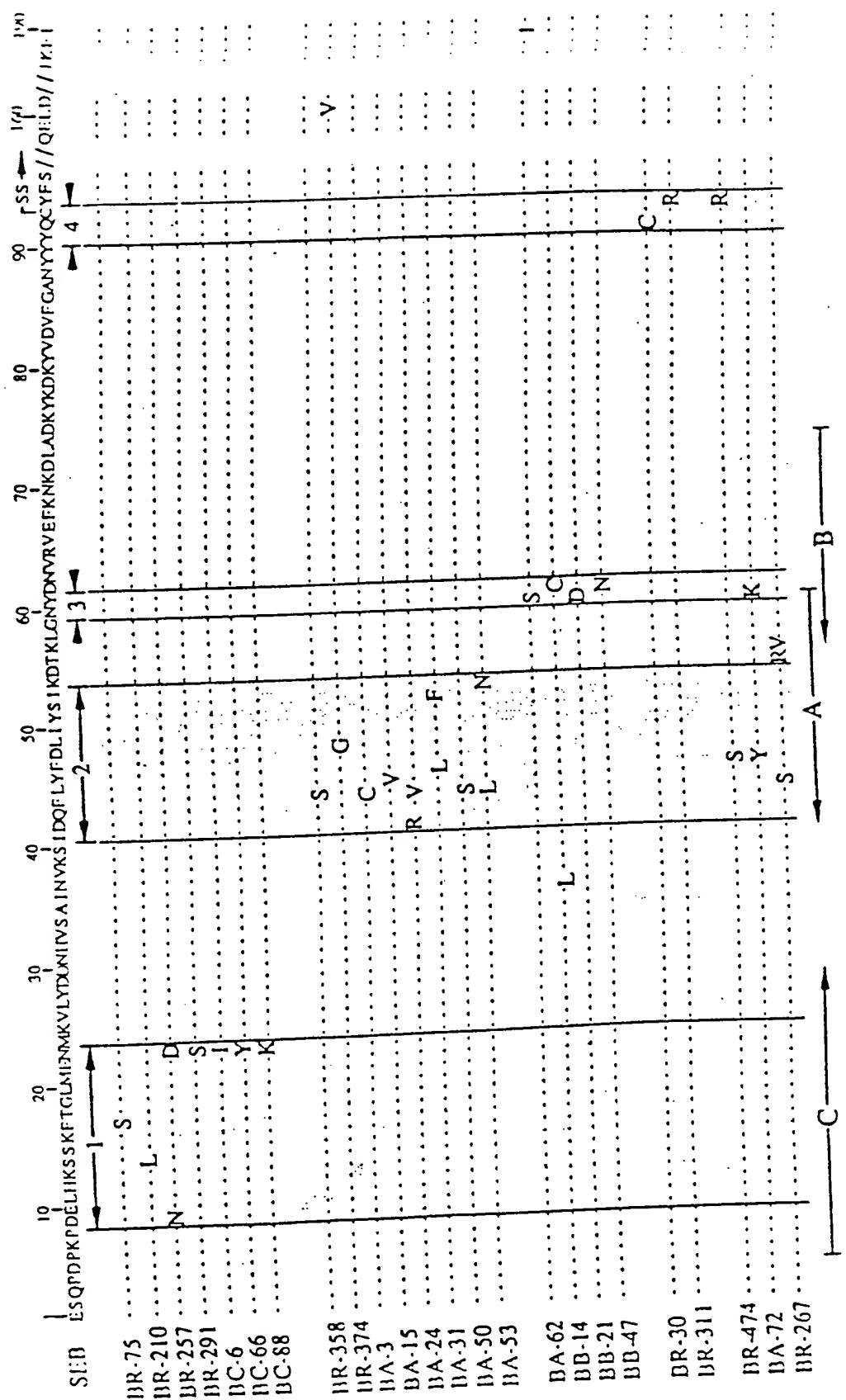


FIG. 2A

FIGURE 3



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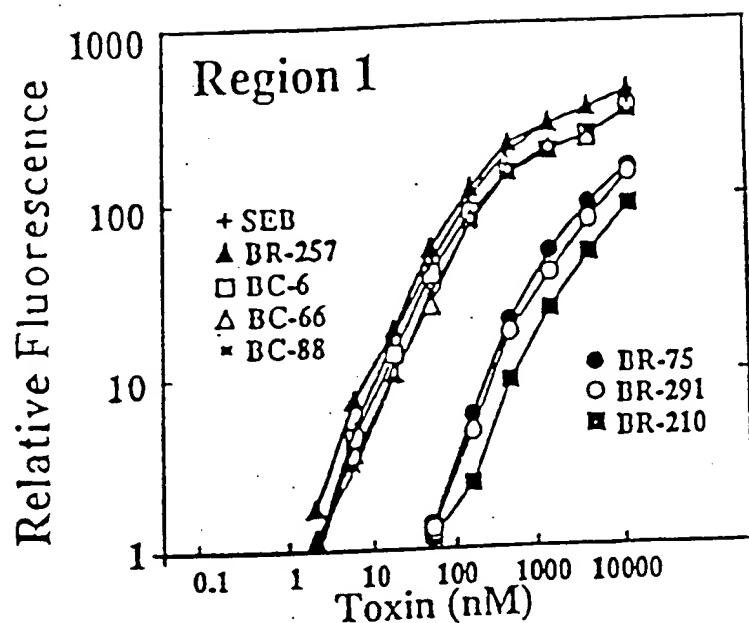


FIG. 4A

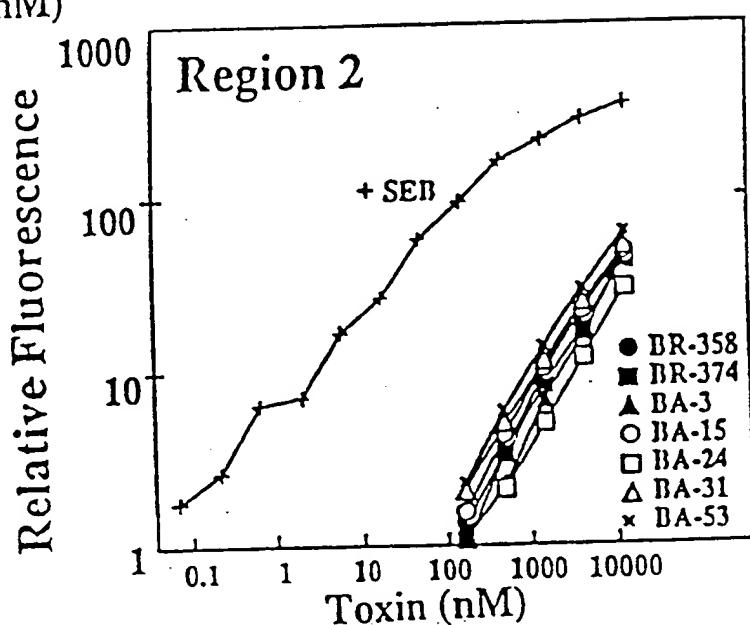


FIG. 4B

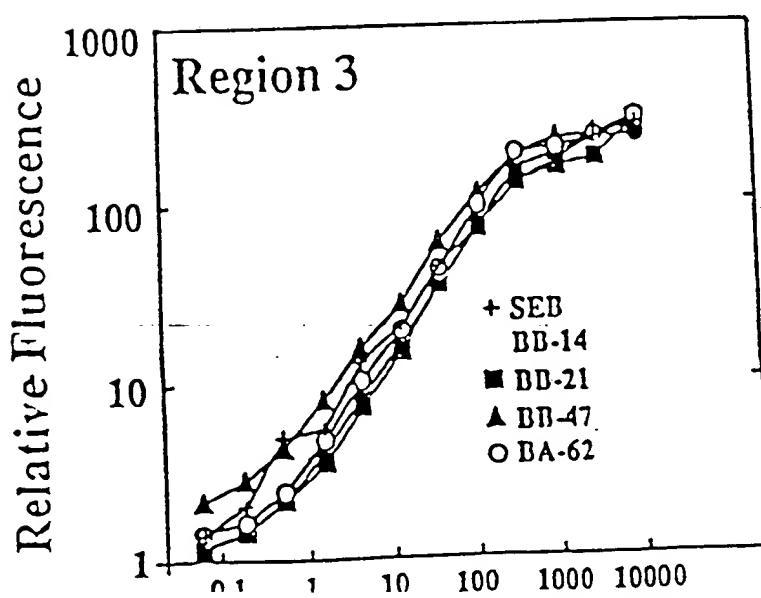


FIG. 4C

5/12

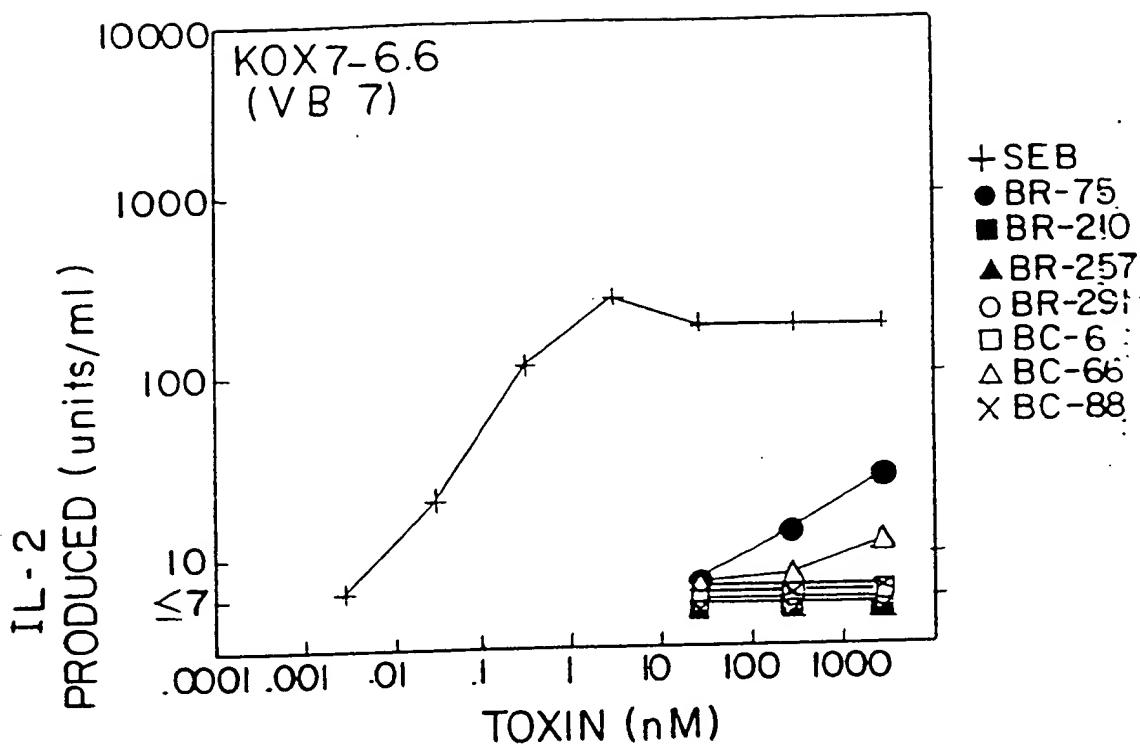


FIG.5A

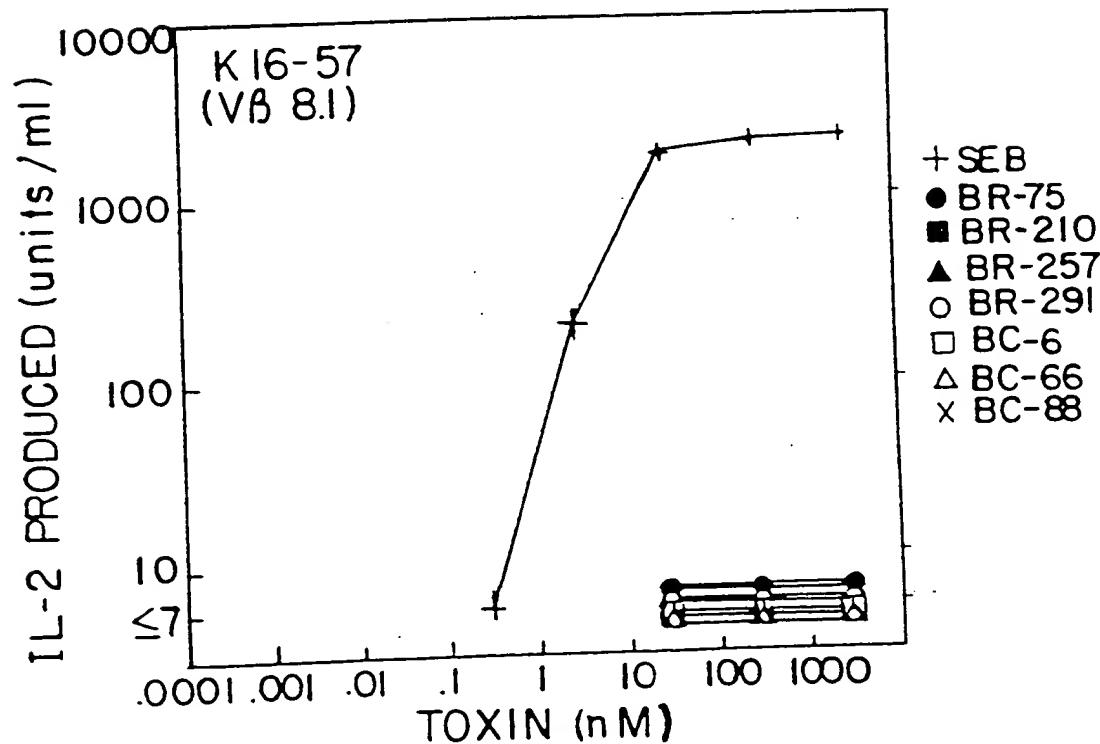


FIG.5B

6/12

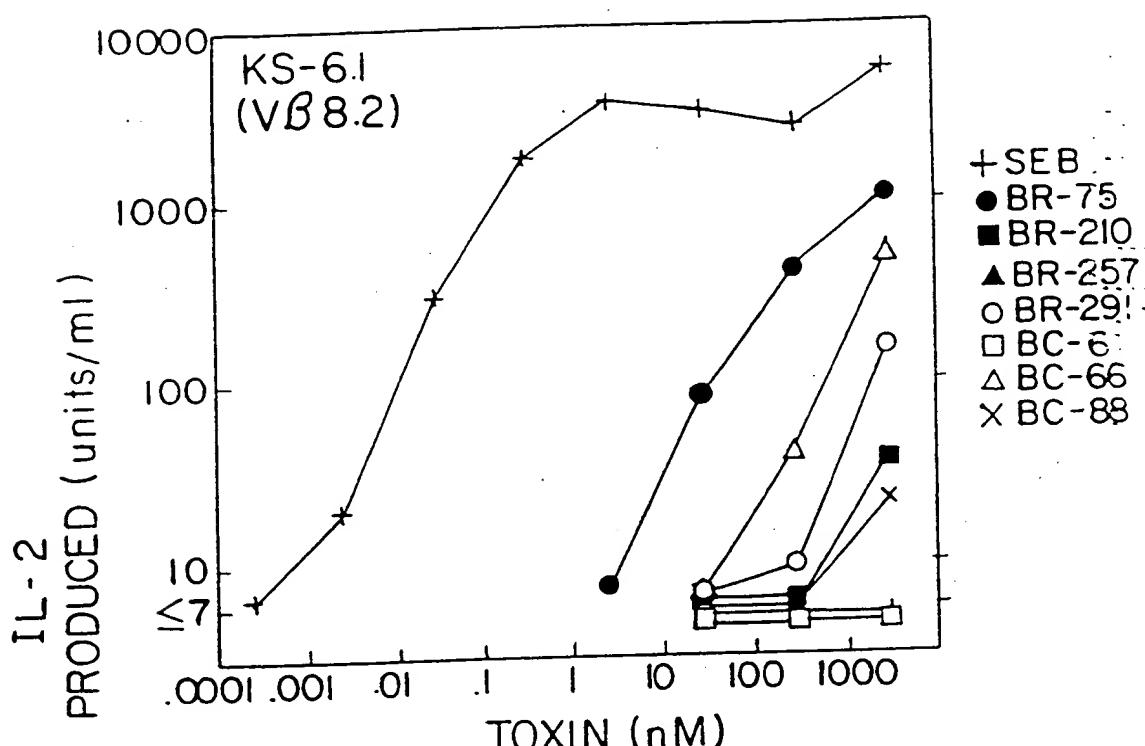
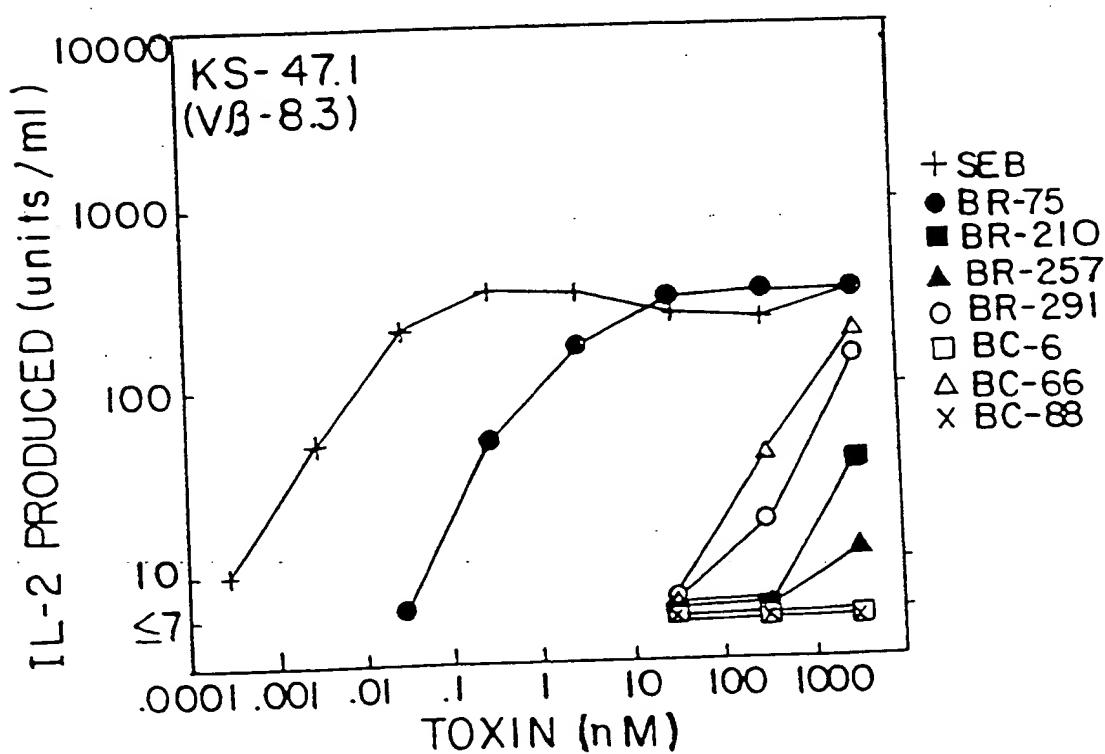


FIG.5C



7/12

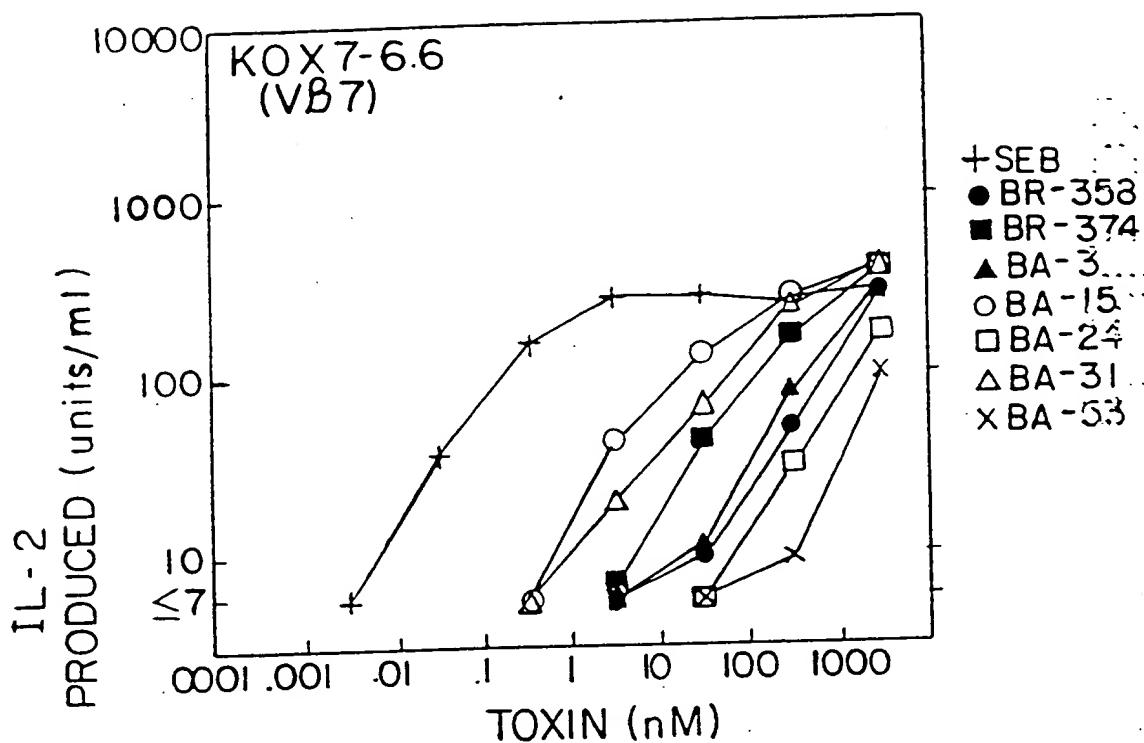
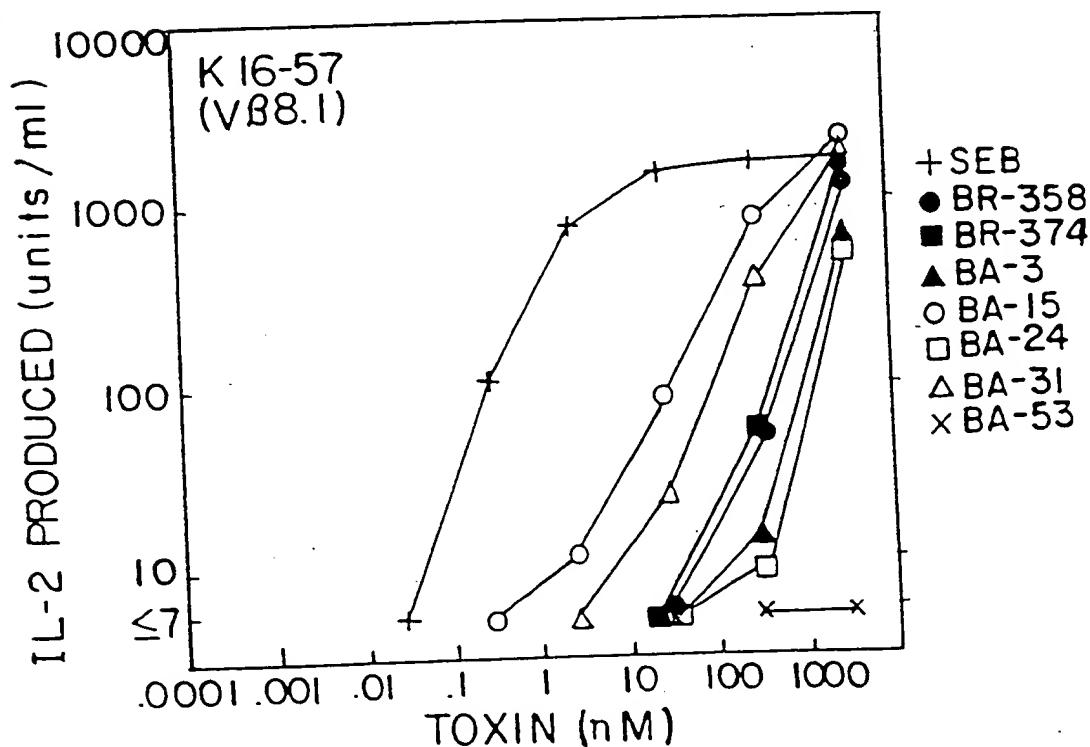


FIG. 6A



8/12

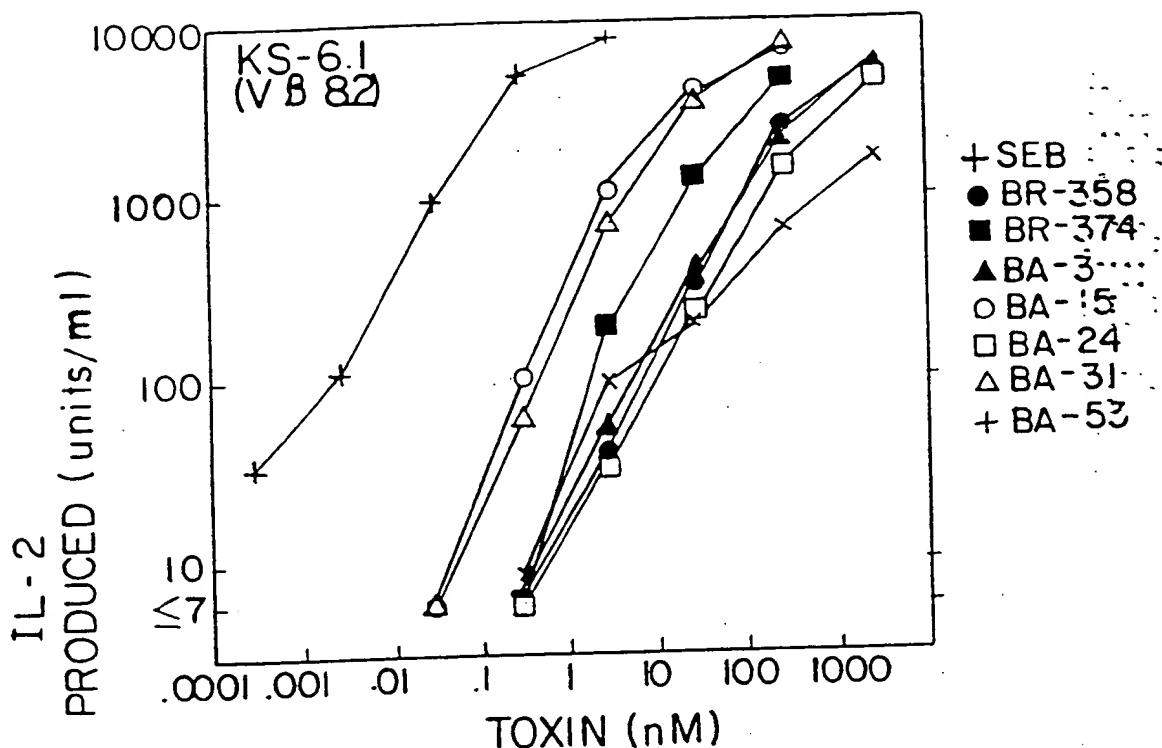
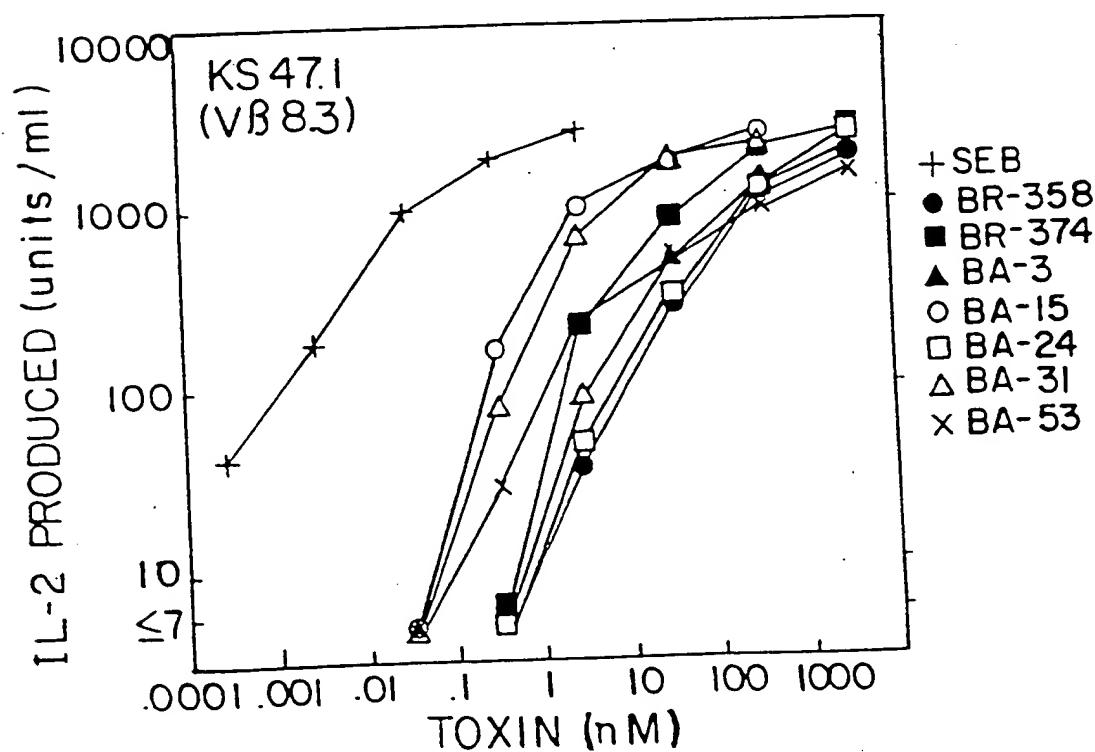


FIG. 6 C



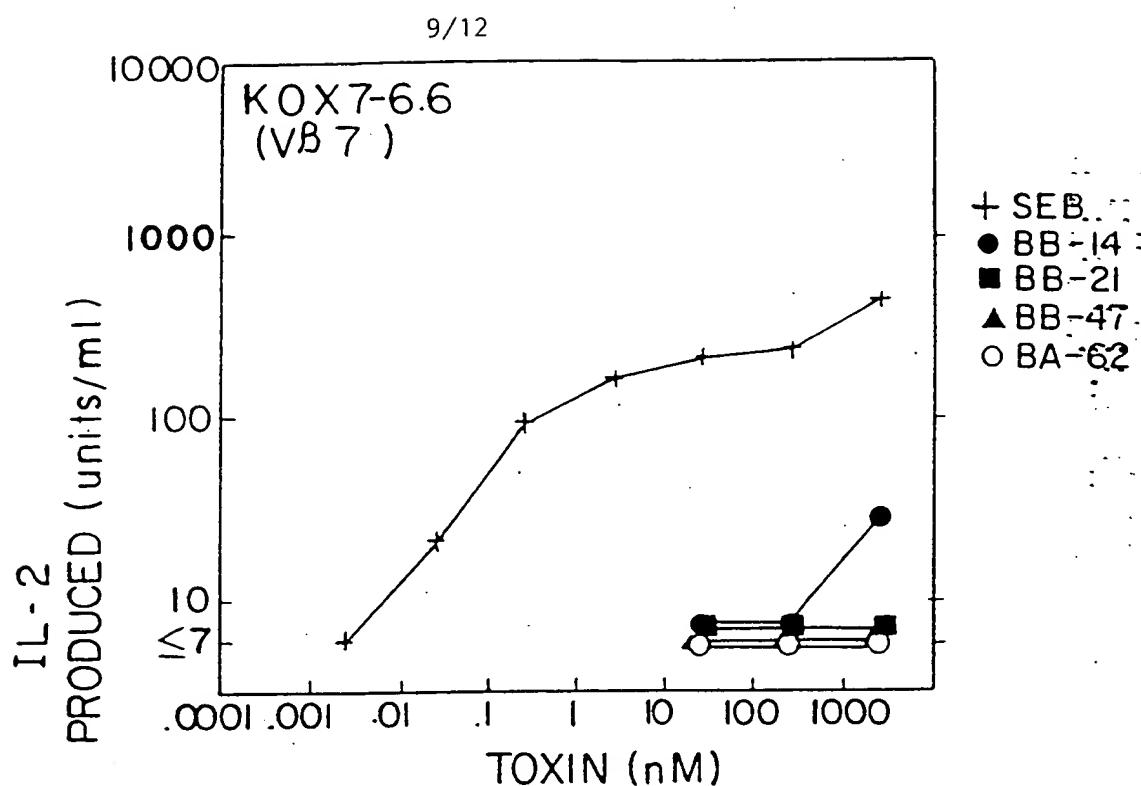


FIG. 7A

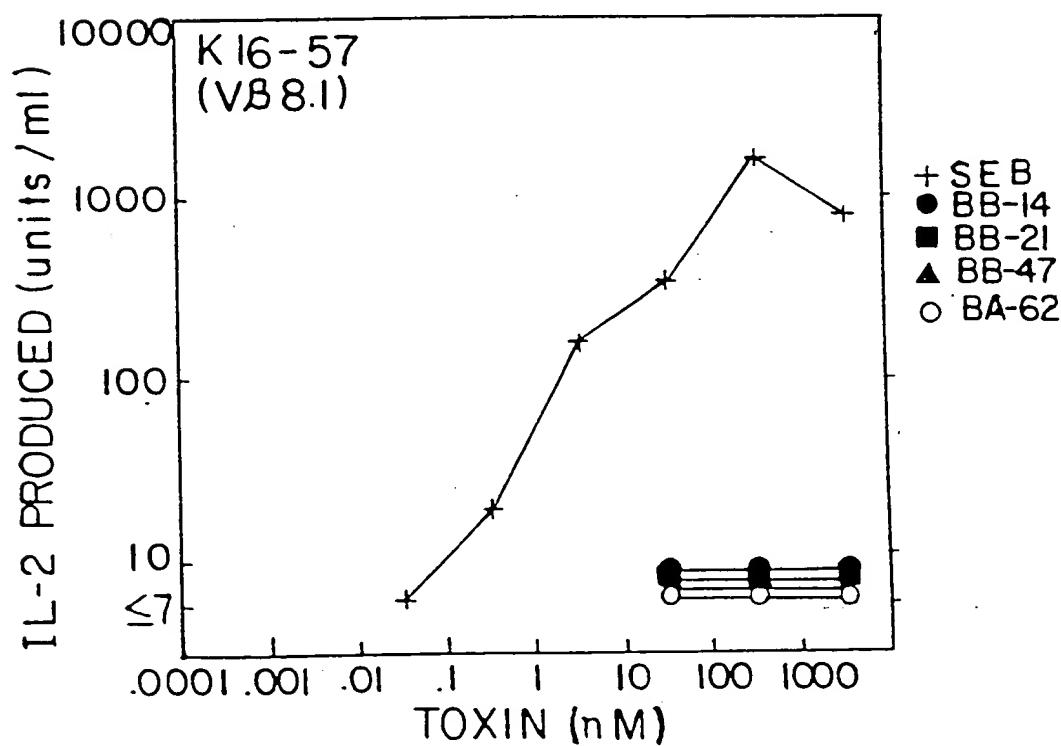


FIG. 7B

10/12

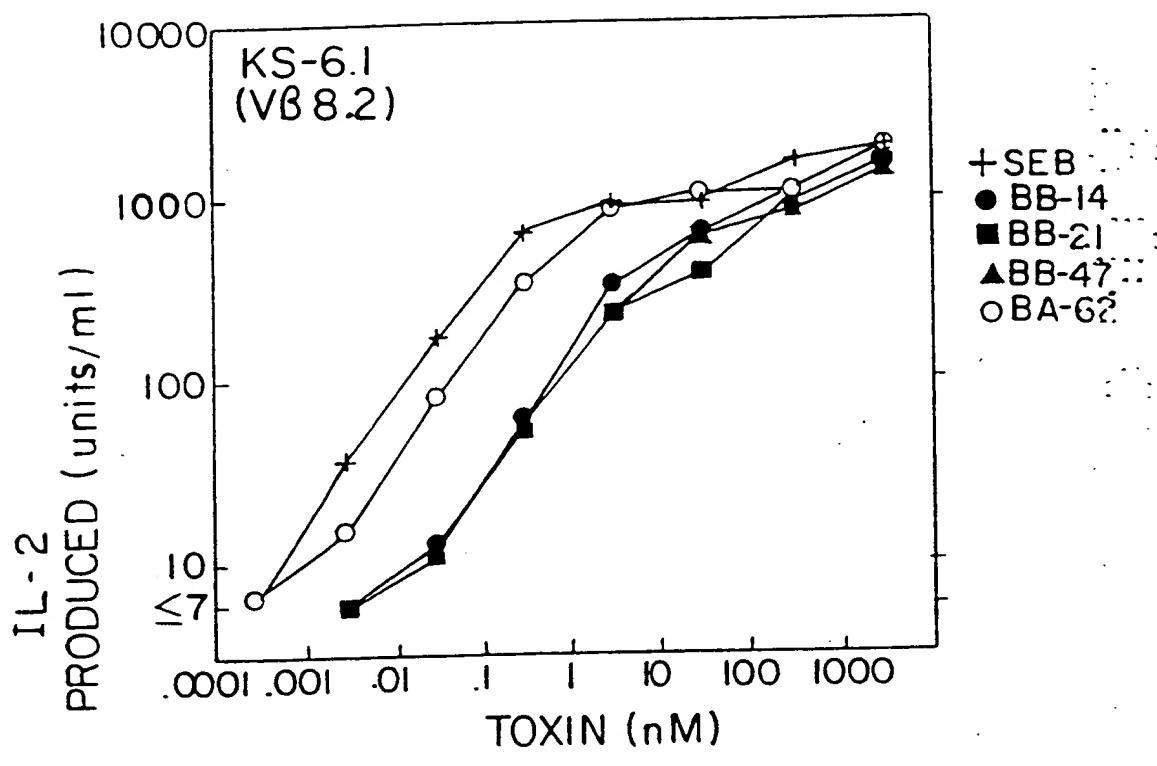
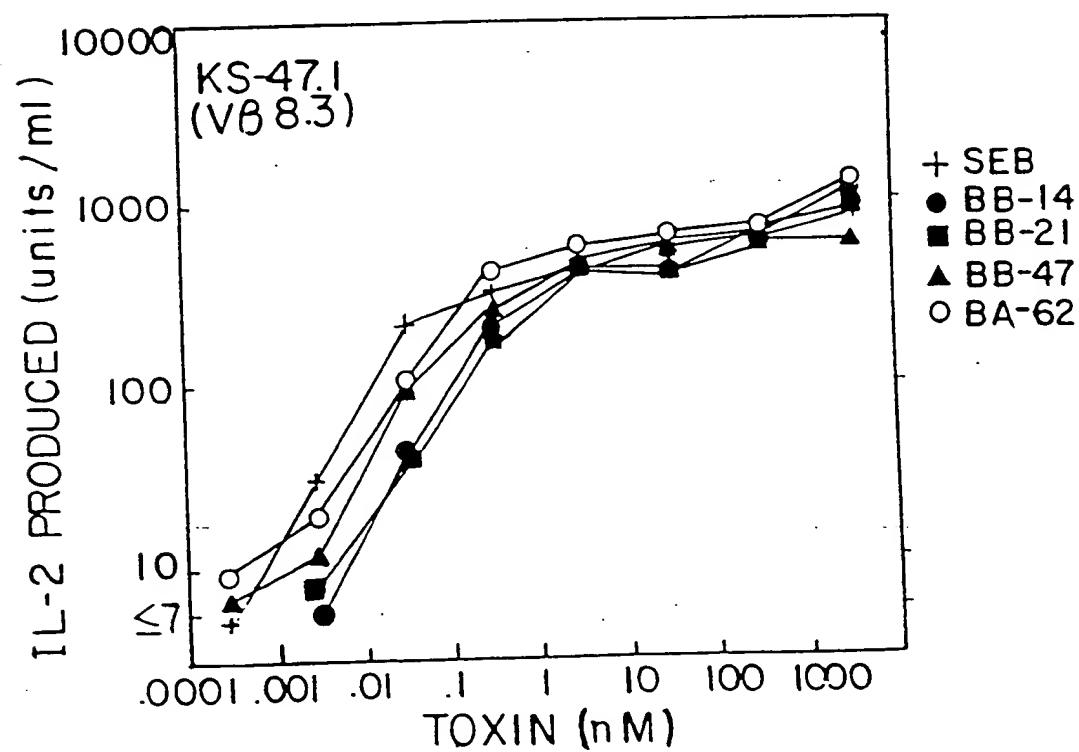


FIG.7C



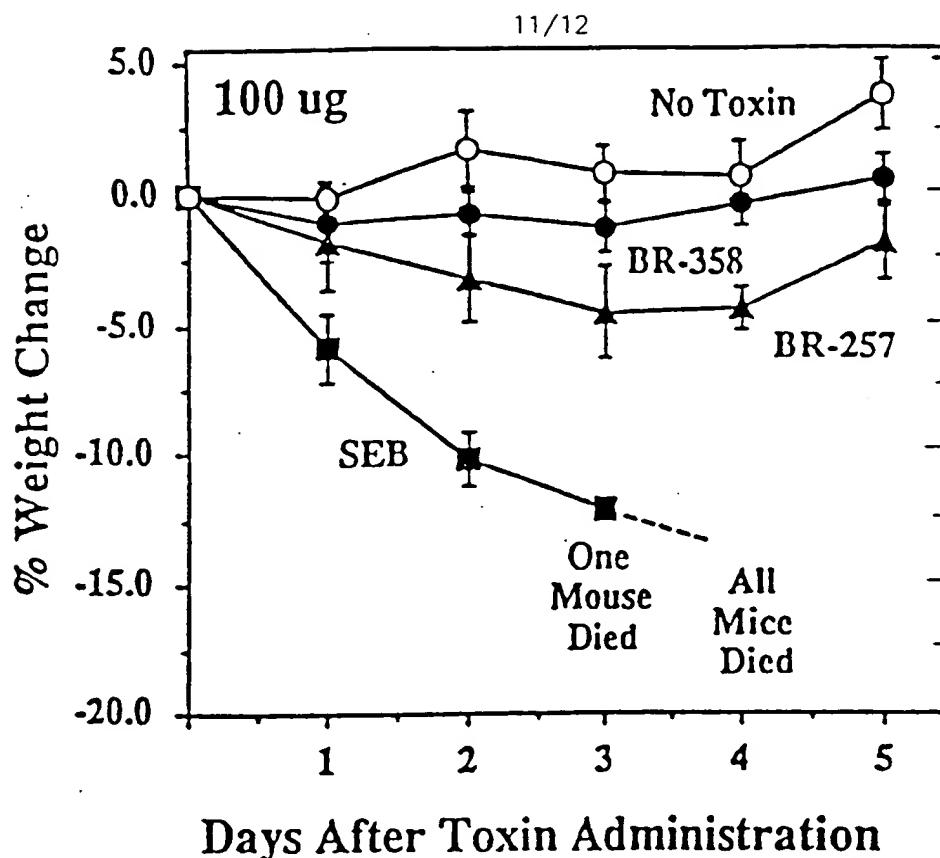
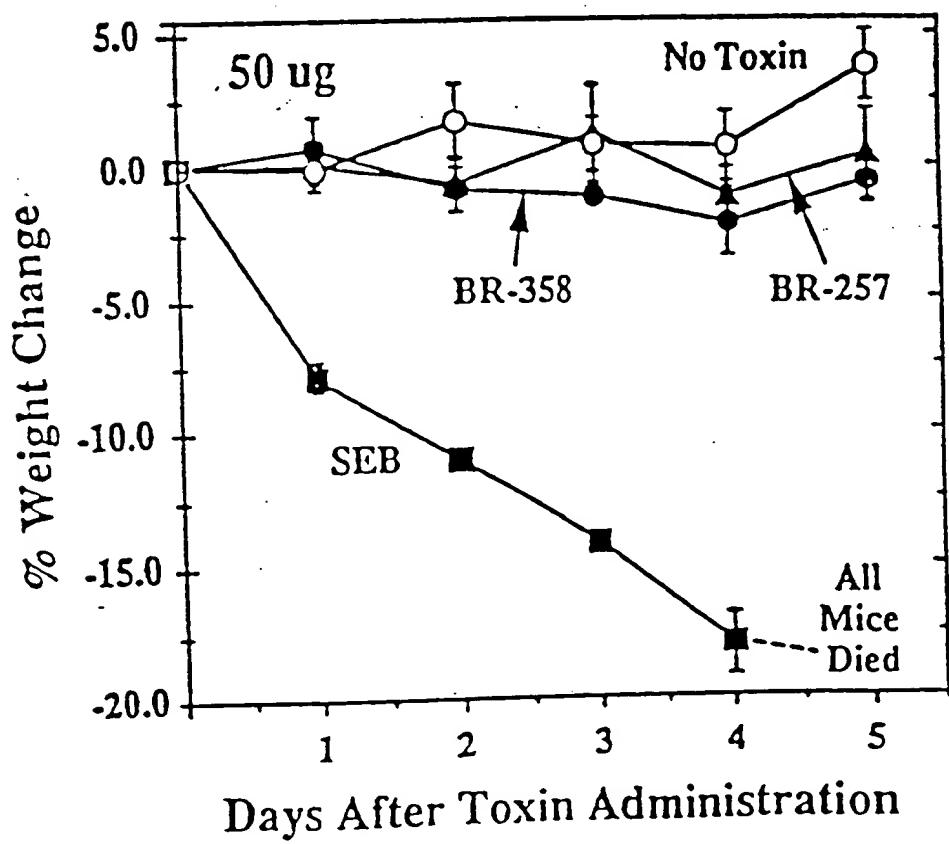
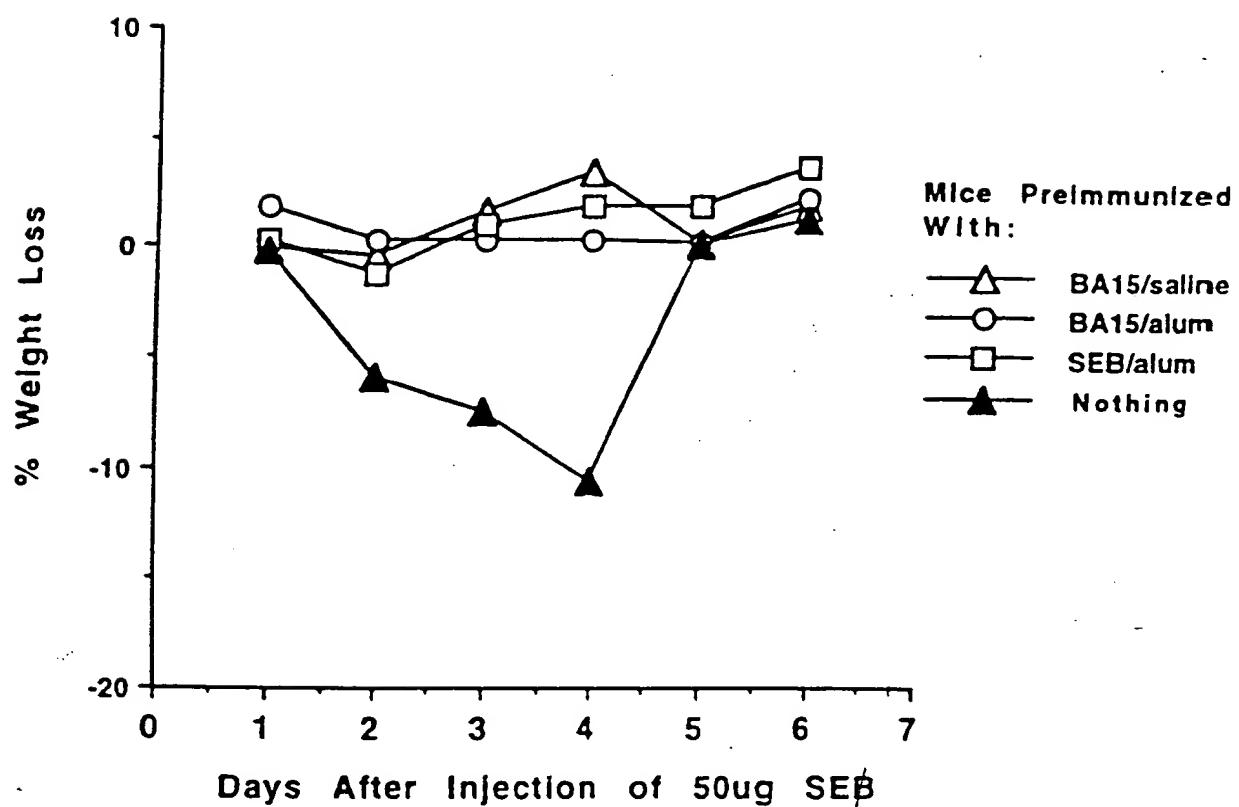


FIG.8A



12/12

FIGURE 9

**MUTANT TOXINS PROTECT AGAINST
CHALLENGE WITH SEB**

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US93/00839

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :Please See Extra Sheet.

US CL :Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/92; 435/7.1, 7.2, 7.33, 69.1, 69.3, 71.1, 172.3, 240.2, 320.1; 514/2; 530/350; 536/22.1, 23.1, 23.2, 23.4, 23.7

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, BIOSIS

search terms: superantigen#, SEB, staphylo?, vbeta, enterotoxin, enterotoxin b

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|-----------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------|
| Y | Jour. Exp. Med., Volume 171, issued February 1990, P. Marrack et al., "The Toxicity of Staphylococcal Enterotoxin B in Mice is Mediated by T Cells", pages 455-464., see entire document. | 1-9 |
| Y | D. M. Glover, "Gene Cloning", published 1984, by Chapman and Hall (N.Y.), see pages 20-47. | 1-9 |
| Y | Jour. Bact., Volume 166, No. 1, issued April 1986, C. L. Jones et al, "Nucleotide Sequence of the Enterotoxin B Gene from <u>Staphylococcus aureus</u> ", pages 29-33, see entire document. | 1-9 |

Further documents are listed in the continuation of Box C. See patent family annex.

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| •E• | earlier document published on or after the international filing date | •Y• | document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art |
| •L• | document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) | •&• | document member of the same patent family |
| •O• | document referring to an oral disclosure, use, exhibition or other events | | |
| •P• | document published prior to the international filing date but later than the priority date claimed | | |

Date of the actual completion of the international search

27 April 1993

Date of mailing of the international search report

04 MAY 1993

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks

Authorized officer

ROBERT A. WAX *Robert A. Wax*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/00839

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (5):

A01 N 37/18, A61K 37/00, 39/02; C07K 3/00, 13/00, 15/00, 17/00; C12N 5/00, 15/00; C12P 21/04, 21/06; C12Q 1/00;

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

424/92; 435/7.1, 7.2, 7.33, 69.1, 69.3, 320.1; 514/2; 530/350; 536/22.1, 23.1, 23.2, 23.4, 23.7

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

European Patent Office
Branch at the Hague

| | |
|------------------------------------------------------------------------------|-----------------------------------------------------------|
| Date of mailing: 23 September 1993 (23.09.93) | in its capacity as elected Office |
| International application No.: PCT/US93/00839 | Applicant's or agent's file reference: SUPRA 223.1/PCT |
| International filing date: 28 January 1993 (28.01.93) | Priority date: 28 January 1992 (28.01.92) |
| Applicant: NATIONAL JEWISH CENTER FOR IMMUNOLOGY AND RESPIRATORY MEDICINE | |

1. The designated Office is hereby notified of its election made:

in the demand filed with the International Preliminary Examining Authority on:

16 August 1993 (16.08.93)

in a notice effecting later election filed with the International Bureau on:

2. The election was

was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Facsimile No.: (41-22) 740.14.35

Authorized officer:

D. Barmes

Telephone No.: (41-22) 730.91.11

6 PATENT COOPERATION TREATY

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NOTIFICATION CONCERNING DOCUMENT TRANSMITTED

From the INTERNATIONAL BUREAU

To:

939614757-7

European Patent Office
Branch at the Hague

Date of mailing:

30 May 1994 (30.05.94)

in its capacity as elected Office

International application No.:

PCT/US93/00839

International filing date:

28 January 1993 (28.01.93)

Applicant:

NATIONAL JEWISH CENTER FOR IMMUNOLOGY AND RESPIRATORY MEDICINE et al

The International Bureau transmits herewith the following documents and number thereof:

copy of the international preliminary examination report (Article 36(3)(a))

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Facsimile No.: (41-22) 740.14.35

Authorised officer:

P. Asseeff

Telephone No.: (41-22) 730.91.11

25
PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

| | | |
|------------------------------------------------------------------------------------------------|------------------------------------------------------------------------------------------------------------------------|---------------------------------------------------|
| Applicant's or agent's file reference SUPRA 223.1/ | FOR FURTHER ACTION See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416) | |
| International application No. PCT/US93/00839 | International filing date (day/month/year) 28 JANUARY 1993 | Priority date (day/month/year) 28 JANUARY 1992 |
| International Patent Classification (IPC) or national classification and IPC (See Attached) | | |
| Applicant NATIONAL JEWISH CENTER FOR IMMUNOLOGY AND RESPIRATORY MEDICINE | | |

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.
2. This REPORT consists of a total of 5 sheets.

This report is also accompanied by ANNEXES, i.e., sheets of the description, claims and/or drawings amended during international preliminary examination and/or containing rectifications made before this Authority.

These annexes consist of a total of 0 sheets.

3. This report contains indications relating to the following items:

- I Basis of the report
- II Priority
- III Non-establishment of report with regard to novelty, inventive step or industrial applicability
- IV Lack of unity of invention
- V Reasoned statement with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
- VI Certain documents cited
- VII Certain defects in the international application
- VIII Certain observations on the international application

| | |
|------------------------------------------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------|
| Date of submission of the demand 16 AUGUST 1993 | Date of completion of this report 21 JANUARY 1994 |
| Name and mailing address of the IPEA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 | Authorized officer ROBERT A. WAX Telephone No. (703) 308-0196 <i>Jill Warden for</i> |
| Facsimile No. NOT APPLICABLE | |

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.

PCT/US93/00839

I. Basis of the report

1. This report has been drawn on the basis of:

 the international application as originally filed. the description, pages (See Attached), as originally filed.

pages _____, filed with the demand.

pages _____, filed with the letter of _____

pages _____, filed with the letter of _____

 the claims, pages 39, as originally filed.pages none, as amended under Article 19.pages none, filed with the demand.

pages _____, filed with the letter of _____

pages _____, filed with the letter of _____

 the drawings, sheets/fig 1-9, as originally filed.sheets/fig none, filed with the demand.

sheets/fig _____, filed with the letter of _____

sheets/fig _____, filed with the letter of _____

2. The amendments have resulted in the cancellation of: pages: nonesheets of drawings/figures No.: none3. This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed, as indicated in the Supplemental Box. Additional observations below.

4. Additional observations, if necessary:

none

II. Priority

1. This report has been established as if no priority had been claimed due to the failure to furnish within the prescribed time limit requested: copy of the earlier application whose priority has been claimed. translation of the earlier application whose priority has been claimed.2. This report has been established as if no priority had been claimed due to the fact that the priority claim has been found invalid.

Thus for the purpose of this report, the international filing date indicated above is considered to be the relevant date.

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. STATEMENT

| | | |
|-------------------------------|-------------|-----|
| Novelty (N) | Claims 1-9 | YES |
| | Claims none | NO |
| Inventive Step (IS) | Claims none | YES |
| | Claims 1-9 | NO |
| Industrial Applicability (IA) | Claims 1-9 | YES |
| | Claims none | NO |

2. CITATIONS AND EXPLANATIONS

EXPLANATION

Claims 1-9 lack inventive step under PCT Article 33(3) over Marrack et al. in view of Jones et al. and Glover.

Marrack et al. teach that mice that have T-cell lose weight when fed *Staphylococcus enterotoxin B* (SEB), whereas mice that lack T-cells do not lose weight when fed SEB, presumably because SEB would be ineffective in the absence of T-cells (p.462, last paragraph). Marrack et al. teach that SEB is a potent T-cell stimulant in mouse or man when it is complexed with class II MHC protein and Vbeta (p. 462, last paragraph).

Marrack et al. fail to teach the DNA sequence of SEB. They also fail to teach making mutants of SEB to obtain mutants that have various effects on the stimulation of T-cells.

Jones et al. teach the DNA sequence of SEB.

Glover teaches methods of making site-directed as well as random mutagenesis.

Given that it is known that SEB is a superantigen that binds Vbeta and stimulates T-cells, as taught by Marrack et al., the skilled artisan would have created mutants of SEB using the DNA sequence disclosed by Jones et al. with the mutagenesis techniques taught by Glover to screen for and obtain mutants that have increased or decreased stimulatory effect, using the weight-loss assay taught by Marrack et al. or other standard techniques using tissue culture of T-cells. The skilled artisan would have then used the mutant superantigen to induce milder reactions so that the wild-type superantigen would be less toxic, with a reasonable expectation of success, since these methods use well-established techniques in the field, with the resultant benefit that understanding SEB function would lead to an antidote to human suffering caused by enterotoxin.

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.

PCT/US93/00839

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

Claims 1-9 are objected to under PCT Article 6 as the disclosure is enabling only for claims limited to the method of using the superantigen that is disclosed in the specification.

Claims 1-5 and 7-9 recite variously "a molecule", "superantigen," "a modified superantigen," "a mutated superantigen." These are not enabled because it would require undue experimentation to determine mutants and modifications that could be made that are related to all of these superantigens. Even if the superantigen were to be narrowed to Staphylococcus superantigens, the thousands of variations and derivations that would be available to this superantigen would be too great and require undue experimentation to determine the functional ones.

Claim 6 recites "modifying T cell response elicited by an antigen". These are vague, and open to interpretation.

Claims 1-3 and 9 are objected to under PCT Article 6 because the disclosure does not teach how to use the composition to administer to patients for treating for the toxic effects of superantigens as claims 1-3 and 9 claim because applicants have not demonstrated that the invention would work in human beings. It is well known in the art that in vitro test results do not necessarily translate to functional application in vivo.

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.

PCT/US93/00839

Supplemental Box

(To be used when the space in any of Boxes I to VIII is not sufficient)

Continuation of Box [No.]: I - VIII

Sheet 9

The International Patent Classification (IPC) and/or the National classification are as listed below:

IPC (5): A01N 37/18, A61K 37/00, 39/02; C07K 3/00, 13/00, 15/00, 17/00; C12N 5/00, 15/00; C12P 21/04, 21/06; C12Q 1/00;

US CL.: 424/92; 435/7.1, 7.2, 7.33, 69.1, 69.3, 320.1; 514/2; 530/350; 536/22.1, 23.1, 23.2, 23.4, 23.7.

I. BASIS OF OPINION

This opinion has been drawn on the basis of the description,

Pages 1-11, 13-38, as originally filed.

Pages none, filed with the demand.

and additional amendments:

Page 12, filed with the letter of 14 June 1993.

'ATENT COOPERATION TR. LTD.

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:
 United States Patent and Trademark
 Office
 (Box PCT)
 Washington D.C. 20231
 United States of America

in its capacity as elected Office

| | |
|-----------------------------------------------------------------------|-----------------------------------------------------------|
| Date of mailing (day/month/year) 29 January 1996 (29.01.96) | Applicant's or agent's file reference Pha-1492-PCT |
| International application No. PCT/SE95/00681 | Priority date (day/month/year) 11 July 1994 (11.07.94) |
| International filing date (day/month/year) 07 June 1995 (07.06.95) | |
| Applicant ABRAHMSÉN, Lars et al | |

1. The designated Office is hereby notified of its election made:

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08 January 1996 (08.01.96)

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made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

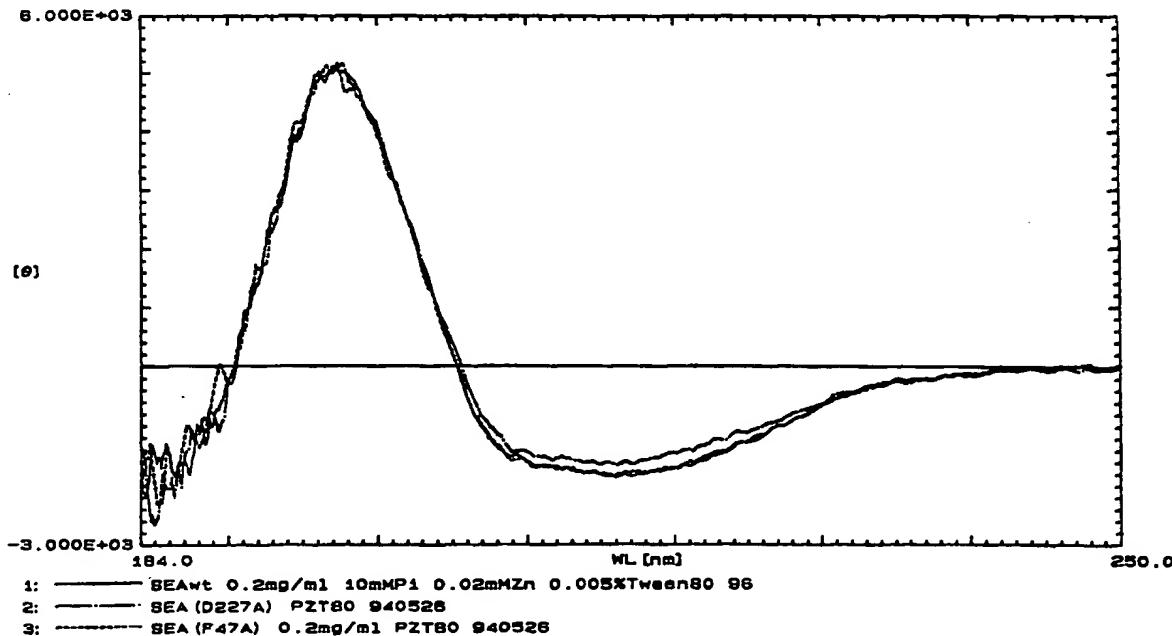
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|---------------------------------------------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------|
| The International Bureau of WIPO 34, chemin des Colombettes 1211 Geneva 20, Switzerland Facsimile No.: (41-22) 740.14.35 | Authorized officer Mirjam Van Straten Telephone No.: (41-22) 730.91.11 |
|---------------------------------------------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------|



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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| (51) International Patent Classification ⁶ : A61K 39/00, 47/48, C07K 16/46, 19/00, 14/31 | | A1 | (11) International Publication Number: WO 96/01650 |
| | | | (43) International Publication Date: 25 January 1996 (25.01.96) |
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| (30) Priority Data: 9402430-4 11 July 1994 (11.07.94) SE | | | |
| (71) Applicant (for all designated States except US): PHARMACIA AB [SE/SE]; S-171 97 Stockholm (SE) | | Published <i>With international search report.</i> | |
| (72) Inventors; and | | | |
| (75) Inventors/Applicants (for US only): ABRAHMSÉN, Lars [SE/SE]; Lillångsgatan 28, S-161 52 Bromma (SE). BJÖRK, Per [SE/SE]; Sigridsgatan 31, S-256 56 Helsingborg (SE). DOHLSTEN, Mikael [SE/SE]; Sotarevägen 16, S-227 30 Lund (SE). KÄLLAND, Terje [SE/SE]; Domherrevägen 4, S-246 32 Löddeköpinge (SE). | | | |
| (74) Agents: BERGANDER, Håkan et al.; Pharmacia AB, Patent Dept., S-751 82 Uppsala (SE). | | | |

(54) Title: A CONJUGATE BETWEEN A MODIFIED SUPERANTIGEN AND A TARGET-SEEKING COMPOUND AND THE USE OF THE CONJUGATE



(57) Abstract

A conjugate comprising (a) a biospecific affinity counterpart (target-seeking group) that binds to a predetermined structure and (b) a peptide that (i) contains an amino acid sequence that is derived from a superantigen, and (ii) has the ability of binding to a V β chain of a T-cell receptor, and (iii) has a modified ability to bind to a MHC class II antigen compared to the superantigen from which the peptide is derived, which parts are covalently linked together.

A CONJUGATE BETWEEN A MODIFIED SUPERANTIGEN AND A TARGET-SEEKING COMPOUND AND THE USE OF THE CONJUGATE.

5 Superantigens are primarily proteins of viral or bacterial origin and are capable of simultaneous binding to MHC class II antigens on mammalian cells and the T cell receptor V β chain. The binding leads to activation of T-lymphocytes and lysis of the MHC class II bearing cells. The moderate degree of polymorphism of the binding part of the V β chain causes a relatively large 10 portion of the T-lymphocytes to be activated when contacted with a superantigen (in comparison with activation through normal antigen-processing).

Initially the superantigen concept was associated with various staphylococcal enterotoxins (SEA, SEB, SEC₁, SEC₂, SED, and SEE). 15 Recently a new staphylococcal enterotoxin named SEH has been discovered (Keyong et al., J. Exp. Med. 180 (1994) 1675-1683). After the interest had been raised, further superantigens were discovered. Examples are Toxic Shock Syndrome Toxin 1 (TSST-1), Exfoliating Toxins (Exft) that are associated with scalded skin 20 syndrome, Streptococcal Pyrogenic Exotoxin A, B and C (SPE A, B, and C), Mouse Mammary Tumor Virus Proteins (MMTV), Streptococcal M Proteins, Clostridial perfringens enterotoxin (CPET) among others. For a review of superantigens and their properties see Kotzin et al. (Adv. Immunol. 54 (1993) 99-166).

25 Pseudomonas exotoxin A has been looked upon as a functional superantigen because there are results indicating that this toxin may be processed intracellularly by accessory cells to fragments that are expressed on the cell surface with the ability to bind to the V β chain and a subsequent activation of T cells. 30 (Pseudomonas exotoxin A. Legaard et al., Cell. Immunol. 135 (1991) 372-382).

35 Superantigens as such have been suggested for therapy of various diseases with curative effects being accomplished through a general activation of the immune system (Kalland et al., WO 9104053; Terman et al., WO 9110680; Terman et al., WO 9324136; Newell et al., Proc. Natl. Acad. Sci. USA 88 (1991) 1074-1078).

**CONFIRMATION
COPY**

In connection with vaccines it has been suggested to use superantigens that have been mutated so as to lose their TCR binding ability (Kappler & Marrack, WO 9314634).

5 The mutation of superantigens has previously been described (Kappler & Marrack, WO 9314634; Kappler et al., J. Exp. Med. 175 (1992) 387-396; Grossman et al., J. Immunol. 147 (1991) 3274-3281; Hufnagle et al., Infect. Immun. 59 (1991) 2126-2134).

We ourselves have previously suggested to employ conjugates between a superantigen and an antibody for therapy in order to 10 lyse cells that express the structure towards which the antibody is directed (Dohlsten et al., WO 9201470; Lando et al., Cancer Immunol. Immunother. 36 (1993) 223-228; Kalland et al., Med. Oncol. Tumor Pharmacother. 10 (1993) 37-47; Lando et al., J. Immunol. 150 (8 part 2) (1993) 114A (Joint Meeting of the 15 American Association of Immunologists and the Clinical Immunology Society, Denver, Colorado, USA, May 21-25 (1993)); Lando et al., Proc. Am. Assoc. Cancer Res. Annu. Meet. 33(0) (1992) 339 (Annual meeting of the American Association for Cancer Research, San Diego, California, USA, May 20-23 (1992)); Dohlsten et al., Proc. 20 Natl. Acad. Sci. USA 88 (1991) 9287-9291). Diseases suggested to be treated have been cancers, viral infections, parasitic infestations, autoimmune diseases and other diseases associated with cells expressing disease-specific surface structures. The 25 experimental work carried out so far has focused on conjugates containing recombinant SEA and various anti-cancer antibodies. The conjugates as such have had a somewhat reduced ability to bind MHC class II antigens compared to the non-conjugated form of the superantigen. It has not been determined if a decreased MHC class II antigen binding ability is beneficial or not for 30 achieving an optimal lyse and an optimal therapeutic effect.

Immune therapy experiments with SEB chemically conjugated to a tumor specific anti-idiotype antibody have previously been described by Ochi et al., (J. Immunol. 151 (1993) 3180-3186).

During the prosecution of the priority application the Swedish 35 Patent Office has additionally cited Buelow et al. (J. Immunol. 148 (1992) 1-6) that describes fusions between Protein A and

fragments of SEB without emphasis of the MHC class II binding or use of the fusion for cell killing; and Hartwig et al. (Int. Immunol. 5 (1993) 869-875) that describes mutations affecting MHC class II binding of the non-fused form of the superantigen 5 streptococcal erythrogenic toxin A.

The objectives of the invention

A first objective of the invention is to improve previously known superantigen-antibody conjugates with respect to general 10 immune stimulation versus directed cytotoxicity. Stimulation results in activated T-lymphocytes and is dependent on the ability of the superantigen to bind to both the T cell receptor and an MHC class II antigen.

A second objective of the invention is to provide conjugates 15 between biospecific affinity counterparts (e.g. antibodies) and superantigens with a modified affinity for MHC class II antigens. This has now been shown to improve the selectivity for 20 superantigen antibody dependent cell cytolysis (SADCC) of cells exposing the antigen (against which the antibody/biospecific affinity counterpart of the conjugate is directed) over other cells exposing MHC class II antigens.

A third objective of the invention is to provide conjugates that can be used as the active principle in the treatment of 25 mammals suffering from cancers, autoimmune diseases, parasitic infestations, viral infections or other diseases associated with cells that on their surface express structures that are specific for respective disease.

The invention

30 The main aspect of the invention is a conjugate comprising
a. a biospecific affinity counterpart that is directed towards
a structure to which one intends to bind to the conjugate,
b. a peptide that
i. is derived from a superantigen,
35 ii. has the ability to bind to the V β chain of the T cell
receptor, and

iii. has a modified ability to bind to MHC class II antigens compared to the superantigen from which the peptide is derived (wild-type of superantigen = SA(wt)).

5 The peptide and the affinity counterpart are covalently linked to each other via a bridge (B).

10 The preferred conjugates have the ability to activate and direct T-lymphocytes to selective lysis of cells that on their surface expose the structure against which the affinity counterpart is directed. This means that the conjugates shall cause cytolysis in an SADCC mediated method (Superantigen Antibody Dependent Cellular Cytotoxicity). See the experimental part below and our previous publications concerning conjugates between superantigens and antibodies (e.g. Dohlsten et al., WO 15 9201470).

15 The inventive conjugates have a structure that is analogous to the superantigen-antibody conjugates described in the prior art (Dohlsten et al., WO 9201470 which hereby is incorporated by reference), i.e. the conjugates complies with the formula:

20 $T-B-SA(m)$

where T represents the biospecific affinity counterpart, SA(m) is the modified superantigen (the above-mentioned peptide), and B is a covalent bridge linking T and SA(m) together.

25 T can in principle be any structure that binds via biospecific affinity. In most important cases, T is capable of binding to a cell surface structure, preferably a disease specific structure as given above. The structure against which T is directed is usually different from (a) the $V\beta$ chain epitope to which the superantigen derived peptide (SA(m)) binds and (b) the MHC class 30 II antigen epitope to which the unmodified superantigen binds. The biospecific affinity counterpart T may primarily be selected among interleukins (e.g. interleukin-2), hormones, antibodies and antigen binding fragments of antibodies, growth factors etc. See for instance Woodworth, Preclinical and Clinical Development of 35 Cytokine Toxins presented at the conference "Molecular Approaches to cancer Immunotherapy", Ashville, North Carolina, November 7-

11, 1993. Polypeptides binding to the constant domains of immunoglobulins (e.g. Proteins A and G and L), lectins, streptavidin, biotin etc were at the priority date considered to be of minor importance.

5 At the priority date, it was preferred that T was an antibody or an antigen binding fragment of an antibody (including Fab, F(ab)₂, Fv, single chain antibody etc), with particular emphasis of an antibody active fragment (such as Fab) of antibodies directed against the so called C242 epitope (Lindholm et al., WO 10 9301303) or against other cancer specific epitopes.

In case T is an antibody it is primarily monoclonal or a mixture of a defined number of monoclonals (e.g. 2, 3, 4, 5 or more). T may be a polyclonal antibody, in case the use is non-therapeutic.

15 It is not imperative for T to have a polypeptide structure.

The modified superantigen SA(m) is primarily a mutated superantigen but may potentially also be a chemically modified superantigen, including fragments of superantigens retaining the ability to bind to the V β chain of the T cell receptor.

20 The expression "mutated superantigen" means that the native ability of the superantigen to bind to MHC class II antigens has been modified on the genomic level by replacing, inserting or removing one or more amino acids in the native superantigen.

25 Superantigen fragments obtained by mutations removing parts of the full amino acid sequence and fragments obtained by enzymatic or chemical cleavage of superantigens may be used equivalently in chemical conjugates of the invention.

30 The modified superantigen SA(m) may comprise one or more amino acid sequences that are derived from different superantigens and that may have been mutated, for instance combinations of the preferred superantigens mentioned below.

The modified superantigen SA(m) as such may exhibit a decreased immunogenicity and toxicity compared to the native superantigen.

35 Other groups/substances that are capable of cross reacting with the V β -chain of the T cell receptor may potentially also be

employed equivalently with the mutated superantigen (SA(m)) as given above. Such groups/substances may be of non-polypeptide structure.

At the end of the priority year the most interesting product 5 candidates of the invention comprised mutated forms of superantigens having multiple MHC class II binding sites and/or the ability to coordinate Zn²⁺, for instance SEA, SED, SEE and SEH.

T as well as SA(m) may be prepared by recombinant techniques.

10 The bridge B may be selected as previously described (Dohlsten et al., WO 9201470), i.e. it shall preferably be hydrophilic and exhibit one or more structure(s) selected among amide, thioether, ether, disulfide etc. In case the bridge have unsubstituted 15 unbroken hydrocarbon chains they preferably lack aromatic rings, such as phenyl. The most important bridges are those obtained by recombinant techniques, i.e. when the conjugation takes places on the genomic level. In such cases oligopeptide bridges containing hydrophilic amino acid residues, such as Gln, Ser, Gly, Glu and Arg, are preferred. Pro and His may also be included. During the 20 priority year it has been decided that the preferred bridge is a peptide comprising three amino acid residues (GlyGlyPro).

The inventive conjugate may comprise one or more modified 25 superantigen(s) per biospecific affinity counterpart and vice versa. This means that T in the formula above may contain one or more modified superantigens in addition to the biospecific counterpart. In analogy SA(m) may contain one or more biospecific affinity counterpart(s) T. The affinity counterpart T and SA(m) may also comprise other structures. The number of modified superantigens per affinity counterpart is preferably one or two.

30 The synthesis of the novel inventive conjugates may be carried out in principle according to two main routes: 1. by recombinant techniques and 2. chemical linking of T to SA(m). The methods are well recognized for the ordinary skilled worker in the field and comprise a large number of variants. It follows that the 35 invention primarily concerns artificial conjugates, i.e. conjugates that are not found in nature.

Chemical linking of a modified superantigen to the biospecific affinity counterpart T often utilizes functional groups (e.g. primary amino groups or carboxy groups) that are present at many positions in each compound. It follows that the final product 5 will contain a mixture of conjugate molecules differing with respect to the position at which linking has taken place.

For recombinant conjugates (fusion proteins) the obtained conjugate substance will be uniform with respect to the linking position. Either the amino terminal of the modified superantigen 10 is linked to the carboxy terminal of the biospecific affinity counterpart or vice versa. For antibodies, such as intact antibodies and antigen binding fragments (Fab, Fv etc), either the light or the heavy chain may be utilized for such fusions. At present time recombinant conjugates are preferred, with 15 preference for Fab fragments and linking of the amino terminal of the modified superantigen to the first constant domain of the heavy antibody chain (CH1), without exclusion of the analogous linking to the light chain or to the VH and VL domain that also may give quite good results.

20 There are two different methods for obtaining large amounts of superantigens (including modified and fused forms) in *E. coli*: intracellular production or secretion. The latter method is preferred for the inventive conjugates because it offers purification of correctly folded protein from the periplasma and 25 from the culture medium. Intracellular production results in a complicated purification procedure and often needs refolding in vitro of the protein (in order for the protein to obtain the correct tertiary structure). The above does not exclude that it is possible to produce active conjugates also in other host 30 cells, e.g. eukaryotic cells, such as yeast or mammalian cells.

The production of mutated superantigens and selection of mutants having a modified ability to bind (affinity) to MHC class II antigens may be carried out according to known techniques (see e.g. Kappler et al., *J. Exp. Med.* 165 (1992) 387-396). See also 35 our experimental part.

The ability of the conjugate to bind to the T cell receptor V β chain, to the target structure and to cause lysis of the target cell depends on i.a. the peptide (SA(m)) that is derived from a superantigen, the biospecific affinity counterpart (T) and the 5 structure and length of the bridge (B). A person ordinary skilled in the art is able to optimize the inventive conjugates with respect to the binding ability and the ability to cause lysis by studying the relationship between effect and structure with the aid of those models that have been disclosed in connection with 10 previously known superantigen antibody conjugates (see the above-referred publications). See also the experimental part below.

By modified ability to bind MHC class II antigens is primarily intended that the ratio IC₅₀(SA(wt)):IC₅₀(SA(m)) is < 0.9 (90 %), such as < 0.5 (< 50 %) and possibly also < 0.01 (< 1 %). In the 15 alternative the modified binding ability of the inventive conjugates can be measured as the ratio of the dissociation constants K_d(SA(wt)):K_d(SA(m)) with K_d measured in nM and with the same limits as for the ratio IC₅₀(SA(wt)):IC₅₀(SA(m)). For the determination of IC₅₀(SA(wt)), IC₅₀(SA(m)), K_d(SA(m)) and 20 K_d(SA(m)) see the experimental part below.

It is previously known that certain superantigens may have two or more sites that bind to MHC class II antigen (Fraser et al., In: Superantigens: A pathogens view on the immune system. Eds. Huber & Palmer, Current Communications in Cell Molecular Biology 25 7 (1993) 7-29). For this type of superantigens the binding ability shall be modified at least one of the binding sites, e.g. as a reduction of the above-mentioned size. Possibly it may suffice with a superantigen modification that create a changed difference in affinity for two MHC class II binding sites, 30 tentatively > 10 % and preferably by reducing the affinity of at least one site.

Superantigens bind to TCR V β chains of different subgroups with varying affinities. In the inventive fusion proteins/conjugates, the superantigen employed may have been 35 modified so as to show an altered subgroup specificity or an altered affinity to one or more members of the subgroup. There

are strong reasons to believe that a parabolic relationship exists between the affinity for TCR V β and stimulation via TCR, i.e. a moderate affinity will give the maximal stimulation. Accordingly an appropriate affinity of a modified superantigen for TCR V β may be at hand as soon as the fusion protein/conjugate comprising the modified superantigen is able to significantly stimulate a resting T cell population representing essentially the distribution of all human V β subgroups to proliferate. The T cell population may be pooled T cells from randomly selected human individuals. By significantly is meant that the stimulation is possible to measure. The results presented in Table II (right column) in the experimental part indicate that the ability to cause SADCC of the inventive conjugates/fusion proteins often is essentially the same as for the fusion comprising the wild-type superantigen.

Main use of the conjugates/fusion proteins of the invention.

The conjugates according to the invention are primarily intended for the treatment of the same diseases as the conjugates between normal superantigens and antibodies. See the above-mentioned publications. Thus the inventive conjugates may be administered either as the main therapy or as adjuvant therapy in connection with surgery or other drugs.

The pharmaceutical composition of the invention comprises formulations that as such are known within the field but now containing our novel conjugate. Thus the compositions may be in the form of a lyophilized particulate material, a sterile or aseptically produced solution, a tablet, an ampoule etc. Vehicles such as water (preferably buffered to a physiologically pH value by for instance PBS) or other inert solid or liquid material may be present. In general terms the compositions are prepared by the conjugate being mixed with, dissolved in, bound to, or otherwise combined with one or more water-soluble or water-insoluble aqueous or non-aqueous vehicles, if necessary together with suitable additives and adjuvants. It is imperative that the vehicles and conditions shall not adversely affect the activity

of the conjugate. Water as such is comprised within the expression vehicles.

Normally the conjugates will be sold and administered in predispensed dosages, each one containing an effective amount of 5 the conjugate that, based on the result now presented, is believed to be within the range of 10 µg - 50 mg. The exact dosage varies from case to case and depends on the patient's weight and age, administration route, type of disease, antibody, superantigen, linkage (-B-) et.

10 The administration routes are those commonly known within the field, i.e. a target cell lysing effective amount or a therapeutically effective amount of a conjugate according to the invention is contacted with the target cells. For the indications specified above this mostly means parenteral administration, such 15 as injection or infusion (subcutaneously, intravenously, intra-arterial, intramuscularly) to a mammal, such as a human being. The conjugate may be administered locally or systemically.

By "target cell lysing effective amount" is contemplated that the amount is effective in activating and directing T-lymphocytes 20 to destroy the target cell.

At the end of the priority year it had been decided that the preferred administration route for conjugates/fusion proteins comprising unmodified superantigens is 3 hours' intravenous infusion per day combined with a fever-reducing agent 25 (paracetamol). The administration is to be repeated during 4 days and stopped before dsecondary antibodies are raised against the fusion protein/conjugate in the patient. This dosage schedule is likely to be applicabple also to the present inventive conjugates/fusion proteins.

30 **Alternative fields of use.**

The inventive conjugates can also be employed to quantitatively or qualitatively detect the structure against which the target-seeking group (T) is directed. In general these 35 methods are well-known to people in the field. Thus, the modified superantigen may function as a marker group within immunoassays

including immunohistochemistry meaning that the marker group in turn is detected by for instance an antibody that is directed towards the peptide (SA(m)) and labelled with an enzyme, isotope, fluorophor or some other marker group known per se. Another 5 immunoassay method is to detect in a cell population cells that on their surface express a structure capable of binding to the target-seeking group (T). This use means that a sample from the cell population is incubated with T-lymphocytes together with the present inventive conjugate as in an SADCC assay. In case the 10 incubation leads to cell lysis this is an indication that the population contains cells that on their surface express the structure.

EXPERIMENTAL PART

15 **MANUFACTURE OF RECOMBINANT PROTEINS**

Antibodies

The experimental work in connection with the invention has primarily been done with monoclonal antibody C215 as a model substance. This antibody is directed against an antigen in the 20 GA-733 family (see for instance EP 376,746) and references cited therein and Larsson et al., Int. J. Canc. 32 (1988) 877-82). The C215 epitope has been judged not to be sufficiently specific for cancer treatment in humans. At the priority date mab C242 (Lindholm et al., WO 9301303) was believed to be a better 25 candidate, as judged from experiments with its fusion product with wild-type SEA.

Bacterial strains and plasmids

The *E. coli* strains UL635 (*xyl-7*, *ara-14*, $T4^R$, Δ ompT) and HB101 30 (Boyer and Roulland-Dessix, J. Mol. Biol. 41 (1969) 459-472) were used for the expression and cloning, respectively. The vector pKP889 was used for expression of Fab-SEA fusion proteins (derived from the murine antibody C215) and the vectors pKP943 and pKP1055 for secretion of SEA (Fig 1). The Fab-SEA expression 35 vector pKP889 is identical to pKP865 (Dohlsten et al, Proc. Natl. Acad. Sci. USA (1994) in press) except that the spacer between

CH1 and SEA is GlyGlyAlaAlaHisTyrGly. Expression from pKP943 yields SEA with the native amino terminus. The use of pKP1055 results in SEA having a Gly residue added at the amino terminus. In both vectors the signals from staphylococcal protein A (Uhlén et al., J. Biol. Chem. 259 (1984) 1695-1702) are used for transcription and translation and a synthetic signal peptide for secretion (L. Abrahmsén, unpublished).

In vitro mutagenesis

Mutations were made by polymerase chain reactions run on a Perkin Elmer Thermocycler. The reaction mixture (100 µl) contained: 1 x PCR buffer from Perkin Elmer Cetus (10 mM Tris/HCl pH 8.3, 1.5 mM MgCl₂, 0.001 % (w/v) gelatine, an additional 2 mM MgCl₂, 0.4 mM dNTPs (Perkin Elmer Cetus), 2.5 units of Ampli Taq DNA polymerase (Perkin Elmer Cetus, USA) and 100 ng DNA template. Primers were added to a final concentration of 0.8 µM. The original template was a plasmid containing *Staphylococcus aureus* enterotoxin A gene identical to the one published by Betley et al. (J. Bacteriol. 170 (1988) 34-41), except that the first codon (encoding Ser) was changed to TCC to furnish a Bam HI site at the 5' end of the gene. Later a derivative containing more unique restriction enzyme sites introduced by silent mutations was used. Mutations introduced next to a restriction site were made with one set of primers, one of these spanning the mutation and the restriction site. For most mutations two set of primers had to be used and the PCR was performed in two consecutive steps. A new restriction enzyme site was introduced together with each mutation to enable facile identification. Oligonucleotides used as primers were synthesized on a Gene Assembler (Pharmacia Biotech AB, Sweden). To confirm each mutation the relevant portion of the nucleotide sequence was determined on an Applied Biosystems DNA-Sequencer using their Taq DyeDeoxy Termination Cycle Sequencing Kit.

Protein production and analysis

E. coli cells harboring the different gene constructs were grown overnight at room temperature (Fab-SEA vectors) and at 24-34°C (secretion vectors, the optimum depends on the mutation).

5 The broth was 2 x YT (16 g/l Bacto trypton, 10 g/l Bacto yeast extract, 5 g/l NaCl) supplemented with kanamycin (50 mg/l). Fusion proteins were induced by addition of isopropyl- β -D-thiogalactoside to a final concentration of 100 μ M. (The protein A promotor used in the expression of non-fused SEA is 10 constitutive). The cells were pelleted at 5000 \times g and the periplasmic contents were released by gently thawing the previously frozen cell pellet in 10 mM Tris-HCl (pH 7.5) on ice during agitation for 1 hour. The periplasmic extracts were 15 clarified by centrifugation at 9500 \times g for 15 minutes. The Fab-SEA proteins were used without further purification. SEA and Gly-SEA were further purified by affinity chromatography on an anti-SEA antibody column. Polyclonal rabbit anti-SEA antibodies were previously collected from rabbits preimmunized with SEA and purified by affinity chromatography on protein G Sepharose® 20 (Pharmacia Biotech).

Protein Analysis

The proteins were separated in precast polyacrylamide SDS Tris-Glycine Novex gels (gradient 4-20 % or homogenous 12 %, 25 Novex novel experimental technology) and either stained with Coomassie Blue or used in Western blot. Polyclonal rabbit anti-SEA antibodies (above) were used to detect SEA in Western blot analysis, followed by porcine anti-rabbit Ig antibodies, and rabbit anti-horseradish peroxidase antibodies and peroxidase. 30 With Fab-SEA fusion proteins peroxidase conjugated rat antibodies recognizing the kappa chain were also used (AAC 08P, Serotech LTD, England). 3,3'-diaminobenzidine (Sigma) was used for visualization of peroxidase.

35 Circular dichroism (CD) spectra were collected in a J-720 spectropolarimeter (JASCO, Japan) at room temperature (22-25°C) in 10 mM phosphate buffer, pH 8.2, with 0.02 mM ZnSO₄ and 0.005 %

(v/v) Tween® 20. The scanning speed was 10 nm/min and each spectrum was averaged from five subsequent scans. The cell path length was 1 mm and the protein concentration 0.2 to 0.5 mg/ml. Guanidine hydrochloride (Gdn-HCl) denaturations at equilibrium were measured at 23°C by CD at 222 nm with a protein concentration of 0.3 mg/ml and a cell path length of 1 mm. These data were used to calculate the apparent fraction of unfolded protein (F_{app}). Equilibrium unfolding parameters were derived by fitting the data to a two-site folding process (Hurle et al., 10 Biochemistry 29 (1990) 4410-4419.

BINDING AND FUNCTIONAL ASSAYS IN VITRO

Materials

Reagents: RPMI 1640 medium obtained from Gibco, Middlesex, 15 England was used. The medium had a pH of 7.4 and contained 2 mM L-glutamine (Gibco, Middlesex, England), 0.01 M HEPES (Biological Industries, Israel), 1 mM NaHCO₃ (Biochrom AG, Germany), 0.1 mg/ml Gentamycin sulphate (Biological Industries, Israel), 1 mM Na-pyruvate (JRH Biosciences Industries, USA), 0.05 mM 20 mercaptoethanol (Sigma Co., USA), 100 times concentrated non-essential amino acids (Flow Laboratories, Scotland) and was supplemented with 10 % fetal bovine serum (Gibco, Middlesex, England). Recombinant SEA(wt), SEA(m) and the fusion products C215Fab-SEA(wt) and C215Fab-SEA(m) were obtained as described 25 above. Human recombinant IL-2 was from Cetus Corp., USA. Mitomycin C was from Sigma Co., USA. Na₂⁵¹CrO₄ was obtained from Merck, Germany. Phosphate buffered saline (PBS) without magnesium and calcium was received from Imperial, England.

Cells: The human colon carcinoma cell line Colo205 and the B cell 30 lymphoma cell line Raji were obtained from American Type Cell Culture Collection (Rockville, MD, USA) (expressing HLA-DR3/w10, -DP7, -DQw1/w2). The EBV-transformed lymphoblastoid B cell line BSM was a generous gift from Dr van De Griend, Dept of Immunology, Dr Daniel den Hoed Cancer Center, Leiden, the 35 Netherlands. The cells were repeatedly tested for mycoplasma

contamination with Gen-Probe Mycoplasma T.C. test, Gen-Probe Inc., San Diego, USA.

SEA activated T cell lines were produced by activation of mononuclear cells from peripheral blood. The blood was received 5 as buffy coats from blood donors at the University Hospital of Lund. The PBMs were stimulated at a concentration of 2×10^6 cells/ml with mitomycin C treated SEA coated BSM cells (preincubated with 100 ng/ml SEA) in medium with 10% FCS. The T cell lines were restimulated biweekly with 20 U/ml human 10 recombinant IL-2 and weekly with mitomycin C treated SEA coated BSM cells. The cell lines were cultivated for 4-12 weeks before being used in the assay.

The viability of the effector cells, as determined by trypan blue exclusion, exceeded 50%.

15

Determination of MHC class II binding characteristics of wild-type and mutant SEA

Radioiodination procedure. Appropriate amounts of wild-type or mutant SEA were radiolabeled with 10 to 25 mCi Na¹²⁵I using 20 enzymobeads with the lactoperoxidase technique (NEN, Boston, MA). The reaction was stopped by quenching with sodium azide and protein-bound radioactivity was separated from free iodine by filtration through a PD-10 column (Pharmacia Biotech AB, Sweden) with R10 medium as elution buffer. Conditions were chosen to 25 obtain a stoichiometric ratio between iodine-125 and protein of \leq 2:1. The radiochemical purity was verified by size-exclusion chromatography on a TSK SW 3000 HPLC column. The effect of the radioiodination on the binding activity was only tested for wild-type SEA and found not to be affected (data not shown).

30 **Direct binding assay.** Raji cells, 6×10^4 /100 μ l, previously cultivated in R10 medium, were added to conical polypropylene tubes and incubated (22°C/45 min) in triplicate with 100 μ l/tube of serially diluted ¹²⁵I-labeled wild-type or mutant SEA. The cells were washed with 2 ml 1% (w/v) bovine serum albumin 35 (BSA) in 10 mM phosphate-buffered saline (PBS), pH 7.4, centrifugated at 300 \times g for 5 minutes and aspirated. This

procedure was repeated twice. Finally, the cells were analyzed for cell-bound radioactivity in a gamma counter (Packard Instruments Co, Downers Grove, IL, USA). The apparent dissociation constant, K_d , and the number of binding sites, N , at 5 saturation were calculated according to Scatchard (Ann. N.Y. Acad. Sci. 51 (1949) 660-72) after subtraction of non-specific binding (i.e. binding after incubation with R10 medium alone).

10 **Inhibition assay** (inhibition of ^{125}I -labeled wild-type SEA binding by mutant SEAs). These inhibition experiments were carried out as is described for the direct binding assay with slight modifications. Briefly, 50 μl of ^{125}I -labeled wild-type SEA was allowed to compete with an excess of unlabeled wild-type or mutant SEA (50 μl /tube) for binding to 6×10^4 /100 μl Raji cells. A tracer concentration yielding $\approx 40\%$ bound radioactivity 15 in the direct assay was used to obtain maximal sensitivity in the inhibition assay. The displacement capacity of the competitor was expressed as the concentration yielding 50 % inhibition (IC₅₀) of bound radioactivity. The binding affinity of the mutants relative to wild-type SEA was calculated using the equation:

20
$$\text{IC}_{50}(\text{SEA(wt)}) : \text{IC}_{50}(\text{SEA(m)})$$

25 In order to analyze whether the mutants compete for binding to the same site on Raji cells as wild-type SEA, the binding data obtained with SEA mutants were plotted as a log-logit function and tested for parallelism with the corresponding data for wild-type SEA.

30 **Inhibition assay** (inhibition of the binding of fluorescent-labeled wild-type SEA by unlabeled wild-type SEA and SEA mutants). Raji cells (2.5×10^5) were incubated with inhibitor (wild-type or mutant SEA; 0-6000 nM) diluted in 50 μl CO₂-independent medium (Gibco) supplemented with 10 % FCS, glutamine and gentamycin at 37°C for 30 minutes. Fluorescein conjugated wild-type SEA was added to a final concentration of 30 nM and the samples were incubated for an additional half hour at 37°C. The samples were washed three times with ice cold PBS supplemented 35 with 1 % BSA (PBS-BSA) and finally kept in 0.4 ml PBS-BSA on ice until they were analyzed. From each sample 10 000 live cells were

analyzed for green fluorescence on a FACStar® (Becton Dickinson) flow cytometer and the mean fluorescence value was calculated using the LYSIS II program.

5 **SDCC and SADCC assays of SEA(wt), SEA(m) and their fusion proteins with C215Fab.**

SDCC-assays. The cytotoxicity of SEA(wt), SEA(m) and their fusions with C215Fab against MHC class II⁺ Raji cells was analyzed in a standard 4 hour $^{51}\text{Cr}^{3+}$ -release assay, using in vitro stimulated SEA specific T cell lines as effector cells. Briefly, ^{51}Cr labeled Raji cells were incubated at 2.5×10^3 cells per 0.2 ml medium (RPMI, 10 % FCS) in microtitre wells at defined effector to target cell ratio in the presence or absence (control) of the additives. Percent specific cytotoxicity was calculated as $100 \times ([\text{cpm experimental release} - \text{cpm background release}] / [\text{cpm total release} - \text{cpm background release}])$. The effector to target cell ratio was 30:1 for unfused SEAs and 40:1 for fusion proteins.

SADCC against of human colon cancer cells. The cytotoxicity of C215Fab-SEA(wt), C215Fab-SEA(m), SEA(wt) and SEA mutants against C215⁺ MHC class II⁻ colon carcinoma cells SW 620 was analyzed in a standard 4 hour $^{51}\text{Cr}^{3+}$ -release assay, using in vitro stimulated SEA specific T cell lines as effector cells. Briefly, $^{51}\text{Cr}^{3+}$ -labeled SW 620 cells were incubated at 2.5×10^3 cells per 0.2 ml medium (RPMI, 10 % FCS) in microtitre wells at effector to target cell ratio 30:1 in the presence or absence (control) of the additives. Percent specific cytotoxicity was calculated as for SDCC assays.

30 **IN VIVO FUNCTIONAL EXPERIMENTS**

Tumor cells. B16-F10 melanoma cells transfected with a cDNA encoding the human tumor associated antigen C215 (B16-C215) (Dohlsten et al., Monoclonal antibody-superantigen fusion proteins: Tumor specific agents for T cell based tumor therapy; 35 Proc. Natl. Acad. Sci. USA, In press, 1994), were grown as adherent cells to subconfluence. The culture medium consisted of

RPMI 1640 (GIBCO, Middlesex, UK) supplemented with 5×10^{-5} β -mercaptoethanol (Sigma, St Louis, MO, USA), 2 mM L-glutamine (GIBCO), 0.01 M Hepes (Biological Industries, Israel) and 10 % fetal calf serum (GIBCO). The cells were detached by a brief 5 incubation in 0.02 % EDTA and suspended in ice cold phosphate buffered saline with 1 % syngeneic mouse serum (vehicle) to 4×10^5 cells/ml.

Animals and animal treatment. The mice were 12-19 weeks old C57B1/6 mice transgeneic for a T cell receptor V β 3 chain 10 (Dohlsten et al., Immunology 79 (1993) 520-527). One hundred thousand B16-C215 tumor cells were injected i.v. in the tail vein in 0.2 ml vehicle. On day 1, 2 and 3, the mice were given i.v. 15 injections of C215Fab-SEA(wt) or C215Fab-SEA(D227A) in 0.2 ml vehicle at doses indicated in the figures 5a and 5b. Control mice were given only vehicle according to the same schedule. On day 21 after tumor cell injection, the mice were killed by cervical dislocation, the lungs removed, fixed in Bouin's solution and the 20 number of lung metastases counted.

RESULTS

"Alanine scanning" of staphylococcal enterotoxin A.

Initially the structure of SEA was unknown and only 25 speculations could be done about what side chains were surface accessible. Therefore, the majority of the mutants were chosen from alignments of homologous superantigens (Marrack and Kappler, Science 248 (1990) 705-711). Conserved (mainly polar) residues were chosen on the rational that some of these superantigens are 30 expected to bind to HLA-DR in a conserved fashion (Chitagumpala et al., J. Immunol. 147 (1991) 3876-3881). Alanine replacements were used according to published strategies (Cunningham and 35 Wells, Science 244 (1988) 1081-1085). During the course of this work the available information increased: i) it was shown that a Zn^{2+} ion is important for the interaction between SEA and MHC class II (HLA-DR) (Fraser et al., Proc. Natl. Acad. Sci. USA 89 (1991) 5507-5511), ii) a mutational analysis of staphylococcal enterotoxin B (SEB) was presented (Kappler et al., J. Exp. Med.

175 (1992) 387-396), and iii) the structure of SEB was presented (Swaminathan et al., Nature 359 (1992) 801-806).

Our first mutant showing a severely reduced affinity for HLA-DR, D227A, was found to co-ordinate the Zn^{2+} ion very poorly (data not shown). Assuming a common fold for SEA and SEB, the new data suggested two MHC class II binding regions; one involving the Zn^{2+} ion and one corresponding to the site defined in SEB. A second set of mutations were made on these assumptions. This second set of mutants were expressed in the form of SEA carrying a glycine added at the amino terminus. First the extension was shown to have no effects on the binding properties of wild-type SEA (next section).

Most of the mutants were expressed and secreted by *E. coli* in a functional form as judged by analysis of the binding of monoclonal antibodies (Table I). Very low amounts were obtained of the mutants E154A/D156A and R160A. Consequently these were excluded from the study. The mutants having an Ala substitution in residues 128, 187, 225 or 227 were not recognized by the monoclonal antibody 1E. The latter two mutants showed a reduced level of expression (more pronounced at 34°C than at 24°C) and migrated faster during SDS-PAGE, under denaturing but not reducing conditions (all other mutants migrated as wild-type SEA, data not shown). As judged by CD spectra analysis the structure of D227A could differ slightly from native SEA (figure 2), but the stability was very close to wild-type SEA (measured as resistance towards guanidine hydrochloride denaturation). The calculated $\Delta\Delta G$ between the mutant and native SEA (SEA(wt)) was -0.16 kcal/mol and is only about 4 % of the ΔG° values (data not shown). Overall the signals in the CD analysis were low, as expected from a mostly β -sheet structure. It was recently reported that His 225 co-ordinates Zn^{2+} (unpublished data in Fraser et al (Proc. Natl. Acad. Sci. USA 89 (1991) 5507-5511). Since Asp 227 is involved in Zn^{2+} co-ordination (above) and presumably located in the same β -sheet as His 225 this suggests that these two residues constitutes the zinc-binding nucleus

found in zinc-co-ordinating proteins (Vallee and Auld, Biochemistry 29 (1990) 5647-5659).

Binding to MHC class II and T cell receptor

5 The MHC class II affinity was calculated from the amounts needed to compete with fluorescein-labeled wild-type SEA for Raji cell exposing large amounts of MHC class II. The displacement capacity of a mutant was calculated from the concentration yielding 50 % inhibition (IC₅₀) of bound fluorescence compared
10 with the concentration needed with wild-type SEA as the competitor. For wild-type SEA and for some mutants, the result from this analysis was compared with the result from an analysis where ¹²⁵I labeled wild-type SEA was used as the tracer. As may be seen in Table II, the values obtained from these two
15 inhibition analyses correlate well

For six selected mutants the binding to MHC class II was measured directly using ¹²⁵I labeled mutant SEA (Table II). With the mutant H50A the values obtained from the direct binding assay and the inhibition assays correlated well but with the mutant
20 F47A a large discrepancy was found: the direct binding indicated only 7 times weaker binding than wild-type SEA but both competition analyses demonstrated around 70 times reduced binding. The data from two of the other mutants indicated two separate binding interactions. For the mutants H225A and D227A
25 the affinity was below the detection limit also in this analysis.

We previously showed that fusion proteins composed of the Fab fragment of a carcinoma reactive antibody and SEA could be used to direct cytotoxic T cells to specifically lyse cancer cells, while the interaction between SEA and the T cell receptor (TCR)
30 was too weak to be detected by itself (Dohlsten et al., Proc. Natl. Acad. Sci. USA, in press). Thus, in contrast to analyses involving the isolated superantigen the Fab fusion context enables a functional assay for the interaction between SEA and the TCR, independent of the MHC class II binding. Consequently,
35 the efficiency of the different conjugates to direct T cells to lyse cells recognized by the Fab moiety was monitored in a

chromium release assay. This analysis confirmed that the mutations shown to affect the MHC class II binding did not affect the TCR binding (Table II).

5 **Biological effects of the mutations**

The proliferative effect was measured as the ability to stimulate peripheral lymphocytes to divide. All three mutants that competes very poorly for MHC class II induced little or no proliferation and the intermediate mutant H187A displayed some 10 proliferative capacity, whereas the other investigated mutants were indistinguishable from the wild-type (table III). Harris et al (Infect. Immun. 61 (1993) 3175-3183) recently reported a similar severe reduction in T cell stimulatory activity for the SEA mutants F47G and L48G. Clearly a strong reduction in any of 15 the two suggested binding regions results in a severe effect on the ability to induce proliferation. This suggests that SEA cross-links two molecules of MHC class II leading to dimerization of the TCR and that this is needed to yield a signal transduction.

20 In contrast the efficiency of the different mutants in directing in vitro stimulated SEA T cells to lyse MHC class II bearing target cells shows correlation with the binding affinity, rather than to the ability to compete (Table III). For example, the efficiency of F47A and D227A are only reduced 2.5 times and 25 300 times, respectively. Thus, here no inherent requirement for divalency too is obvious. The increase in multivalency resulting from the significantly larger number of TCRs on the surface of activated T cells might partially shield the effect of a lower avidity in the SEA/MHC class II interaction. That dimerization is 30 not needed to direct T cell cytotoxicity has previously been demonstrated by the use of carcinoma specific bifunctional antibodies containing one anti-CD3 moiety and one anti-carcinoma moiety (Renner et al., Science 264 (1994) 833-35).

35 **In vivo functional experiments:** The results are represented in figures 6a and 6b. Treatment of mice with C215Fab-SEA(wt) and

C215Fab-SEA(D227A) were both highly effective in reducing the number of lung metastases of B16-C215 melanoma cells. The therapeutic effect was essentially identical for the two variants of the targeted superantigens. Treatment with C215Fab-SEA(wt) 5 resulted in 70 % lethality at doses of 5 μ g/injection. In contrast, no mice died when the same dose of C215Fab-SEA(D227A) were used. Taken together, SEA(D227A) is an example of a mutant with reduced toxicity and retained therapeutic efficiency when incorporated in a Fab-SEA fusion protein.

10

DISCUSSION

The structure of the complex between SEB and HLA-DR was recently reported (Jardetzky et al., Nature 368 (1994) 711-718). Most of the SEB residues identified to be involved in this 15 interaction are conserved in SEA. Our data on mutant D227A indicates a weak affinity for the interaction between this site of SEA (the amino proximal site) and the MHC class II, having a K_d value higher than 8 μ M. The K_d for the interaction between SEB and HLA-DR was recently reported to be 1.7 μ M (Seth et al., 20 Nature 369 (1994) 324-27). The different interactions between SEB, TCR and HLA-DR were investigated and it was shown that the complex between SEB and HLA-DR was not stably maintained in the absence of TCR. Plasmon resonance experiments indicated that this was because of a very fast off-rate. The avidity effects obtained 25 if SEA cross-links two molecules of MHC class II followed by a subsequent dimerization of the TCR could explain how SEA may induce proliferative effects at concentrations well below the K_d . Assuming that the mutation F47A reduces the affinity of the amino proximal site below significance, the K_d of the Zn^{2+} site is 30 around 95 nM. This hypothesis was recently strengthened by the observation that the mutants F47R, F47R/H50A and F47R/L48A/H50D show identical affinity for MHC class II as F47A (unpublished).

Based on the SEB structure (Kappler et al., J. Exp. Med. 175 (1992) 387-396) and on homology alignments (Marrack and Kappler, 35 Science 248 (1990) 705-711), it is strongly suggested that His225 and Asp227 are located in the same β -sheet and thus the side

chains could be proximal. Thus, most likely these two residues constitute the zinc-binding nucleus found in zinc-co-ordinating proteins (Vallee and Auld, Biochemistry 29 (1990) 5647-5659). Similarly to these mutants, the mutants with a replacement at residue 128 or 187 are also recognized by all monoclonals except 1E. Fraser et al (Proc. Natl. Acad. Sci. USA 89 (1991) 5507-5511) showed that Zn^{2+} is bound to SEA and is needed for a high affinity interaction with MHC class II. The affinity for zinc was not affected by the addition of HLA-DR. Based on this observation and the high affinity for Zn^{2+} (K_d of around 1 μ M) a co-ordination exclusively provided by SEA and involving 4 fold co-ordination was suggested. Our data indicates an involvement of the four residues N128, H187, H225 and D227. The function of the former two residues is not yet clear; instead of providing a ligand N128 could help in the deprotonation of D227. One argument for this is that the effect of replacing D227 is more severe than when replacing H225.

It was previously reported that there is a lack of correlation between the affinity of different superantigens for the MHC class II and the capacity to stimulate T cells to proliferate (Chintagumpala et al., J. Immunol. 147 (1991) 3876-3881). These results might partly be explained by different affinities of the superantigens towards different TCR $V\beta$ -chains. Here we have observed the same lack of correlation but in contrast to separate superantigens the mutants display identical TCR affinity as shown in the Fab-SEA context (measured as SADCC). The most likely explanation for the lack of correlation is that two binding regions identified in this analysis represent two separate binding sites that yields not only a co-operative binding, but which results in the cross-linking of two molecules of MHC class II, which in turn yields dimerization of two molecules of the T cell receptor. This would imply that the affinity of both sites are important to obtain the proliferative effect. A high avidity results from the interactions within a hexameric complex involving two molecules of SEA, TCR and MHC class II. Thus the

strong affinity/avidity of SEA towards MHC class II enables SEA interaction with the TCR despite a low direct affinity.

Other biospecific affinity counterparts: A fusion protein of SEA(D227A) and an IgG-binding domain of staphylococcal protein A has been produced by recombinant technology and expressed in *E. coli*. This reagent has successfully been used to target T-lymphocytes to Mot 4 and CCRF-CEM cells (obtained from ATCC) that are CD7 and CD38 positive but HLA-DP, -DQ and -DR negative. The Mot 4 and CCRF-CEM cells were preincubated with anti-CD7 or anti-CD38 mouse monoclonals (Dianova, Hamburg, Germany). In order to enhance binding between the mouse monoclonals and the IgG-binding part of the fusion protein rabbit anti-mouse Ig antibody was also added.

In comparison with protein A-SEA(wt), protein A-SEA(D227A) had a decreased ability to bind to Daudi cells expressing MHC class II antigen.

Table I

Confirmation of mutant structural integrity. The binding of six monoclonal antibodies was monitored.

| Mutation | Monoclonal antibody | | | | | |
|-------------|---------------------|----|-----|----|----|-------|
| | 1A | 2A | 3A | 1E | 4E | EC-A1 |
| Wild-type | + | + | + | + | + | + |
| D11A/K14A | + | + | + | + | + | + |
| D45A | + | + | + | + | + | + |
| F47A | + | + | + | + | + | + |
| H50A | (+) | + | (+) | + | + | + |
| K55A | + | + | + | + | + | + |
| H114A | + | + | + | + | + | + |
| K123A/D132G | + | + | + | + | + | + |
| N128A | + | + | + | - | + | + |
| K147A/K148A | + | + | + | + | - | + |
| E154A/D156A | ND | ND | ND | + | ND | ND |
| R160A | ND | ND | ND | + | ND | ND |
| H187A | + | + | + | - | + | + |

25

| | | | | | | |
|-------------|---|---|---|---|---|---|
| E191A/N195A | + | + | + | + | + | + |
| D197A | + | + | + | + | + | + |
| H225A | + | + | + | - | + | + |
| D227A | + | + | + | - | + | + |

5 Footnotes: A plus sign indicates binding, parenthesis indicate 50 to 90 % binding compared with wild-type SEA. ND means not determined.

Table II

10 Binding of SEA mutants to the MHC class II and the T cell receptor. The latter was monitored as the ability to direct activated cytotoxic T-cells specifically to lyse carcinoma cells using Fab-SEA fusions of the different mutants (SADCC).

| Mutation | IC ₅₀ (nM) SEA-FITC ¹ | IC ₅₀ (nM) 125I-SEA ¹ | K _d (nM) 125I labeled ¹ | SADCC (% of wild-type ¹) |
|-------------|------------------------------------------------|------------------------------------------------|--------------------------------------------------|-----------------------------------------|
| wild-type | 50 | 38 | 13 | 100 ² |
| Gly-SEA | 50 | ND | ND | 100 ² |
| D11A/K14A | 50 | ND | ND | ND |
| D45A | 53 | ND | ND | ND |
| F47A | 3150 | 2943 | 95 | 100 |
| H50A | 150 | 132 | 32 | 100 |
| K55A | 44 | ND | ND | ND |
| H114A | 48 | ND | ND | ND |
| K123A/D132G | 188 | 75 | 12/237 | 100 |
| N128A | 1150 | ND | 2.9/76 | 100 |
| K147A/K148A | 58 | ND | ND | ND |
| H187A | 1030 | 602 | 97 | 100 |
| E191A/N195A | 51 | ND | ND | ND |
| D197A | 78 | ND | ND | ND |
| H225A | >9000 | 9600 | ND | ND |
| D227A | >9000 | >10000 | >8000 | 100 |

Footnotes: 1) ND means not determined. 2) In the Fab-SEA context the spacer between C_H1 and SEA ends with a Gly.

Table III

Biological effects of the mutations. The ability to stimulate resting T cells to proliferate and the ability to direct cytotoxic cells to lyse MHC class II exposing target cells were monitored (SDCC = Superantigen Dependent mediated Cellular Cytotoxicity).

| | Mutation | Proliferation % | SDCC |
|----|-------------|--------------------|-----------------------------|
| | | | EC ₅₀ (relative) |
| 10 | wild-type | 100 | 1 |
| | Gly-SEA | ND | 1 |
| | D11A/K14A | ND | 0.8 |
| | D45A | 50 | 1.3 |
| | F47A | <0.2 | 2.5 |
| 15 | H50A | 20 | 1.4 |
| | K55A | 100 | 1.3 |
| | H114A | ND | 1 |
| | K123A/D132G | 40 | 2.1 |
| | N128A | 40 | 1.2 |
| 20 | K147A/K148A | ND | 0.7 |
| | E154A/D156A | ND | ND |
| | R160A | ND | ND |
| | H187A | 15 | 4 |
| | E191A/N195A | 100 | 1.1 |
| 25 | D197A | ND | 1.3 |
| | H225A | <0.2 | 3x10 ² |
| | D227A | <0.01 | 3x10 ² |

Footnotes: ND means not determined.

30 **LEGENDS TO THE FIGURES**

General: The mutant SEA(D227A) (=SEA(m9) or mutant m9) was at the priority date the most promising SEA variant. We have therefore selected to present in vitro and in vivo results with this variant (Figures 3-6).

Figure 1.

Schematic outline of the plasmids used to express SEA and C215Fab-SEA. The coding regions and the two transcription terminators following the product genes are indicated by boxes.

5 The gene encoding the kanamycin resistance protein is labeled Km. lacI is the lac repressor gene. V_H and C_{H1} indicates the gene encoding the F_d fragment of the heavy chain of the murine antibody C215. Likewise V_K and C_K indicates the gene encoding the kappa chain. Rop is the gene encoding the replication control 10 protein from pBR322. The promoters directing transcription of product genes are shown as arrows, in pKP889 the trc promoter and in the other two vectors the promoter from staphylococcal protein A (spa). The region containing the origin of replication is indicated by ori. The only difference between SEA encoded by 15 pKP943 and pKP1055 is a glycine residue added at the N-terminus of the latter. The SEA gene contained in the latter vector also contains more unique restriction enzyme sites, introduced by silent mutations.

Figure 2

20 Circular dichroism spectra for wild-type SEA and for the mutants F47A and D227A, representing the most severely reduced mutations in each MHC class II binding region. The solid line is the curve for wild-type SEA. The curves for the mutants are dotted or center, F47A respectively D227A.

25 **Figure 3** shows the concentration dependency of superantigen dependent mediated cellular cytotoxicity (SDCC) for SEA(wt) and SEA(D227A).

Figure 4 shows the concentration dependency of superantigen dependent cell mediated cytotoxicity (SDCC) for C215Fab-SEA(wt) 30 and C215Fab-SEA(D227A).

Figure 5 shows the concentration dependency of superantigen mAb dependent cell mediated cytotoxicity (SADCC) for C215Fab-SEA(wt) and C215Fab-SEA(D227A) compared to free SEA(wt).

35 **Figure 6a** compares the therapeutic effects obtained in C57B1/6 mice carrying lung metastases of B16-C215 melanoma cells by treatment with C215Fab-SEA(wt) and C215Fab-SEA(D227A).

Figure 6b shows toxicity of C215-SEA(wt) and C215-SEA(D227A) for the treatments represented in figure 6a.

P A T E N T C L A I M S

1. Conjugate comprising
 - a. a biospecific affinity counterpart (target-seeking group) that is capable of binding to a predetermined structure, and
 - b. a peptide that
 - i. contains an amino acid sequence that is derived from a superantigen,
 - ii. has the ability to bind to a V β chain of a T cell receptor, and
 - iii. has a modified ability to bind to MHC class II antigens compared to the superantigen from which the peptide is derived,
- 15 which parts are covalently linked together.
2. The conjugate according to claim 1, **characterized** in that
 - a. the biospecific affinity counterpart is directed towards a cell surface structure, and that
 - 20 b. the conjugate has the ability to activate T-lymphocytes to lyse cells that exhibit the cell surface structure on their surface.
- 25 3. The conjugate according to any one of claims 1-2, **characterized** in that the biospecific affinity counterpart is an antibody or an antigen binding fragment of an antibody.
- 30 4. The conjugate according to any one of claims 1-3, **characterized** in that it is a fusion protein.
5. The conjugate according to any one of claims 1-4, **characterized** in that the peptide is a mutated superantigen.
- 35 6. The conjugate according to any one of claims 1-5,

characterized in that the peptide is derived from a superantigen and that its ability to bind to MHC class II antigens is altered with at least 10 %.

- 5 7. The conjugate according to any one of claims 1-6, **characterized** in that the superantigen is staphylococcal enterotoxin A, B, C₁, C₂, D, or E.
- 10 8. The conjugate according to claim 7, **characterized** in that the superantigen in addition may be derived from staphylococcal enterotoxin H
- 15 9. The conjugate according to any one of claims 1-8, **characterized** in that the structure against which the biospecific affinity counterpart is directed is a structure that is expressed on the cell surface during a disease, for instance a cancer, a viral infection, an autoimmune disease or a parasitic infestation.
- 20 10. A method for the lysis of mammalian cells, **characterized** in that the cells are contacted with T-lymphocytes and a conjugate according to any one of claims 2-9 in which the biospecific affinity counterpart is directed against a surface structure on the cells that are to be lysed, said incubation being performed under conditions allowing for lyse of said cells.
- 25 11. A method for selective lysis of cells (I) that are present together with other cells (II) and that express a structure that is preferentially occurring on those cells (I) that are to be lysed, **characterized** in that the cells (I) together with (II) simultaneously are contacted with a conjugate according to any one of claims 2-9 in which the biospecific affinity counterpart is directed towards a surface structure on the cells (I) that are to be lysed, said contact being performed under conditions permitting lysis.

12. A method according to claim 11, **characterized** in that the cells (I) are associated with diseased conditions, such as a cancer, a viral infection, a parasitic infestation, an
5 autoimmune disease etc.
13. A method for the treatment of a diseased condition of a mammal, which condition means the presence of specific cells that are associated with the condition by the expression of a disease specific surface structure, **characterized** in that one administers to the mammal a therapeutically effective amount of a conjugate according to any one of claims 2-9 in which conjugate the biospecific affinity counterpart is directed against the disease specific structure.
10

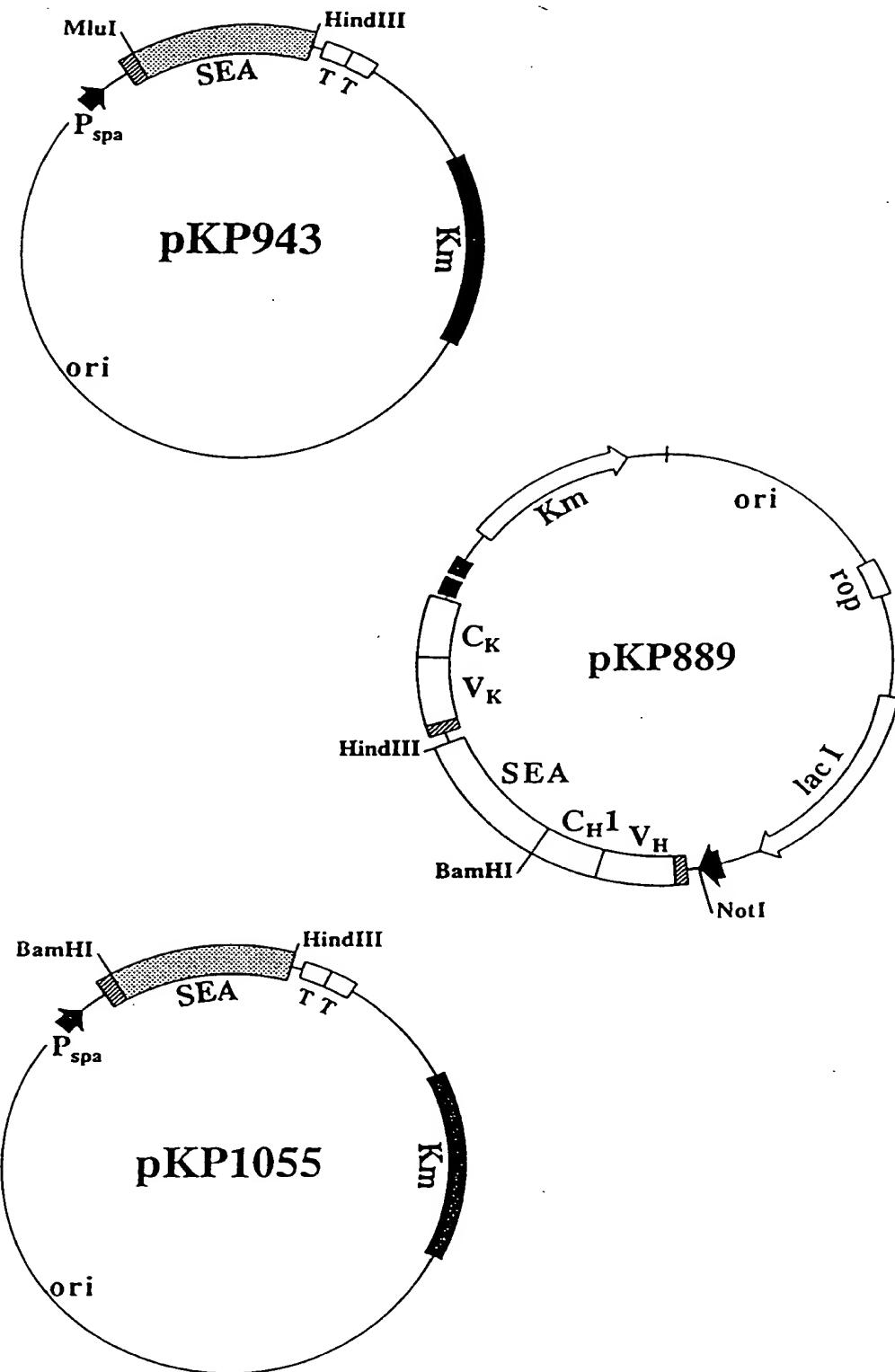
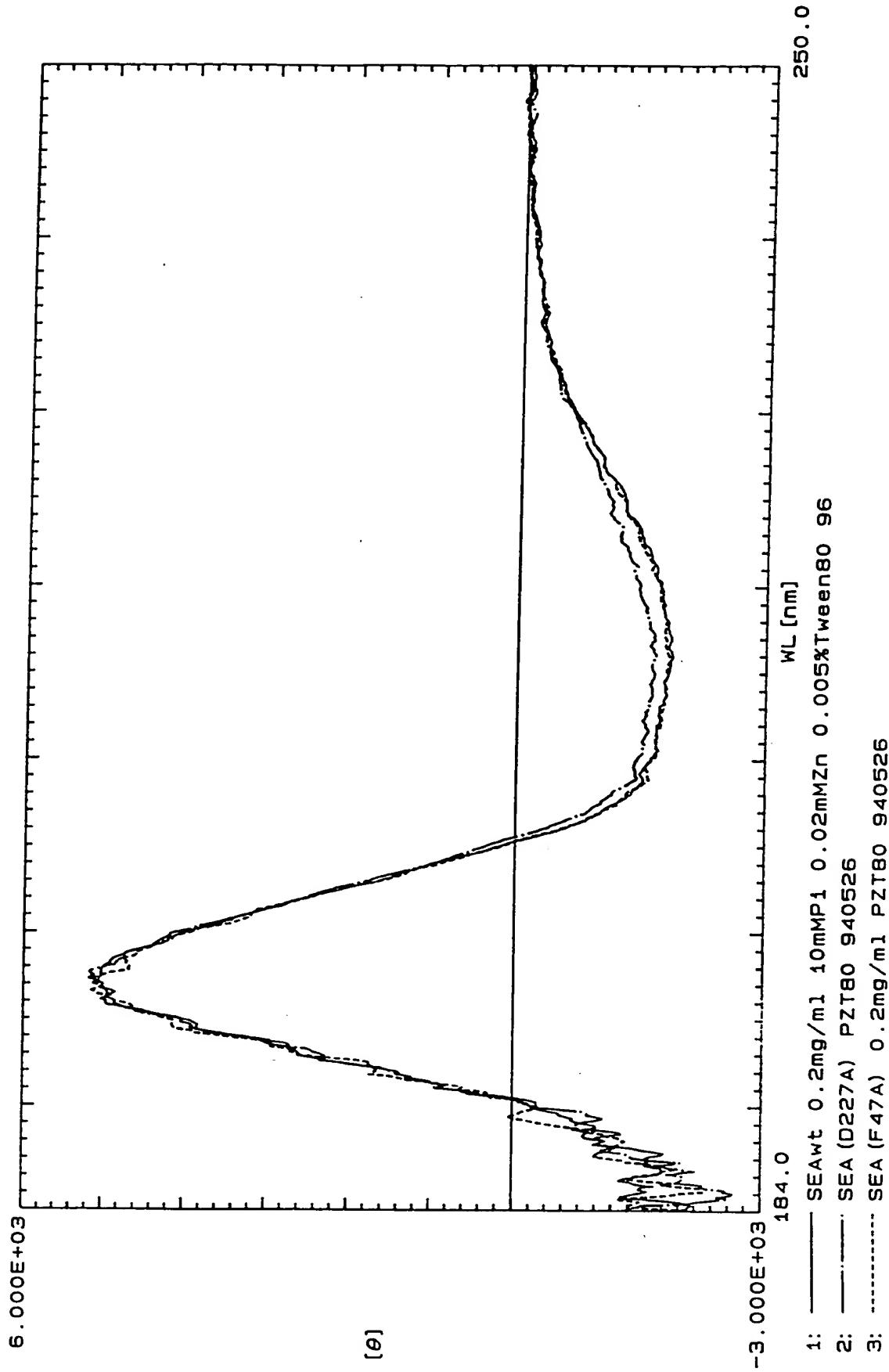


FIGURE 1

FIGURE 2



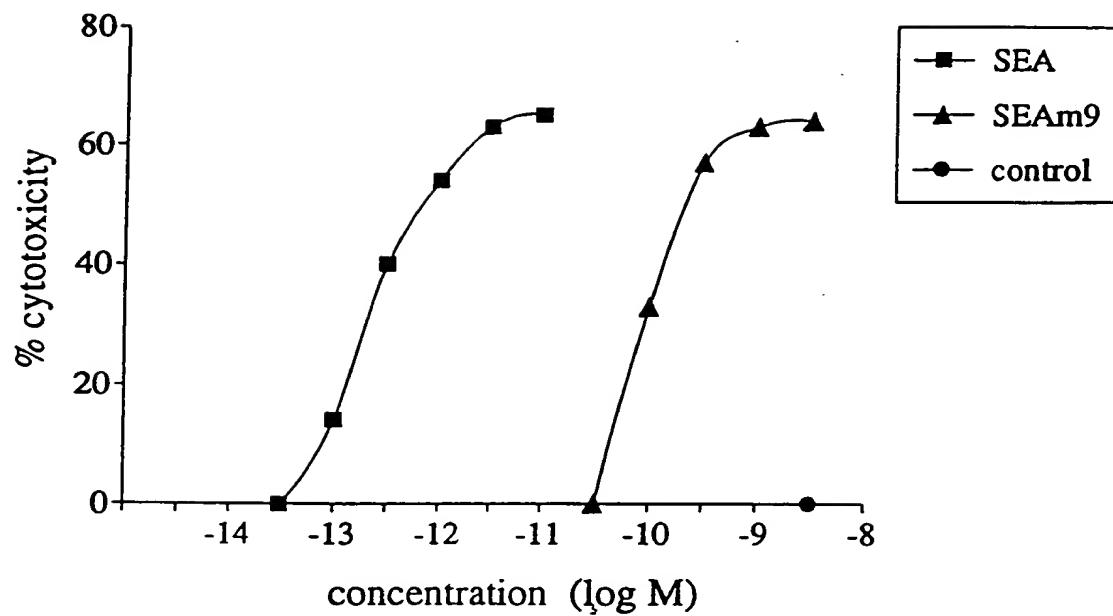


FIGURE 3

4/5

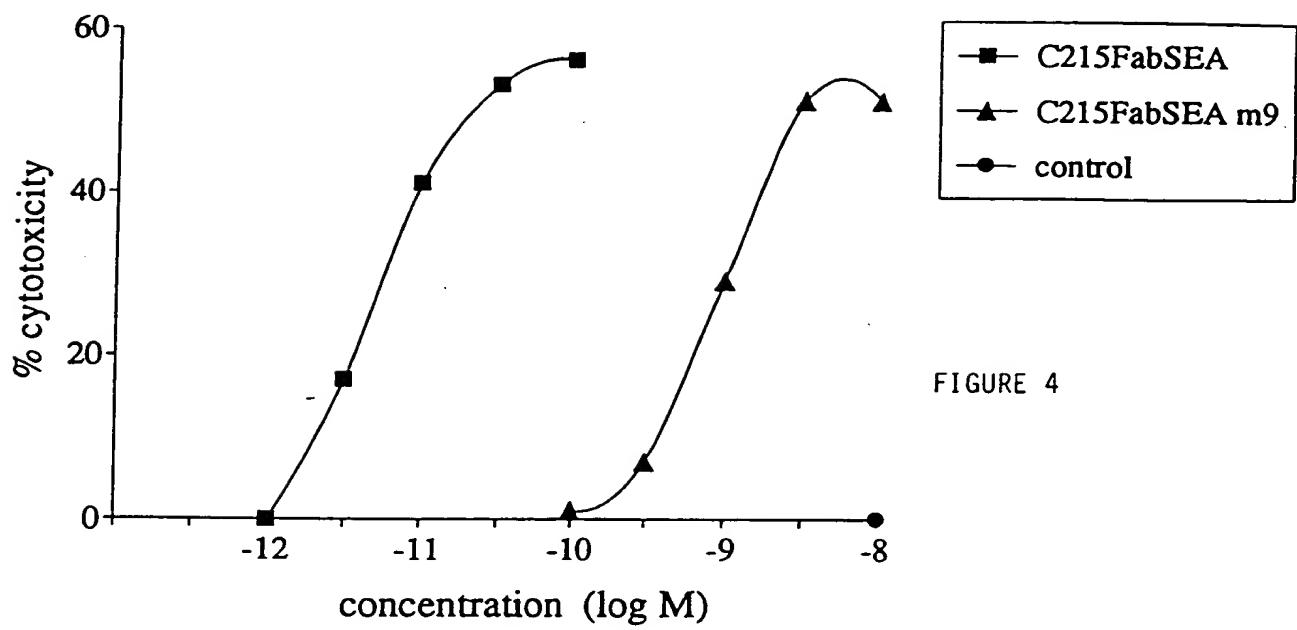


FIGURE 4

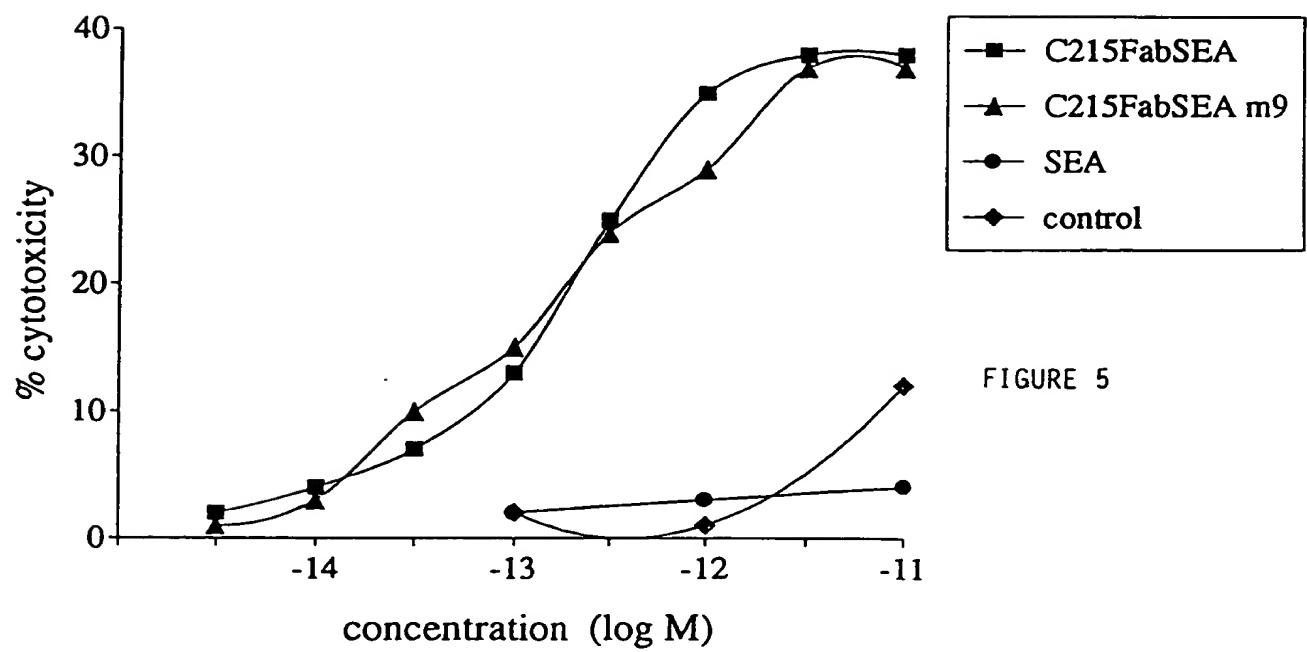


FIGURE 5

Therapeutic effect

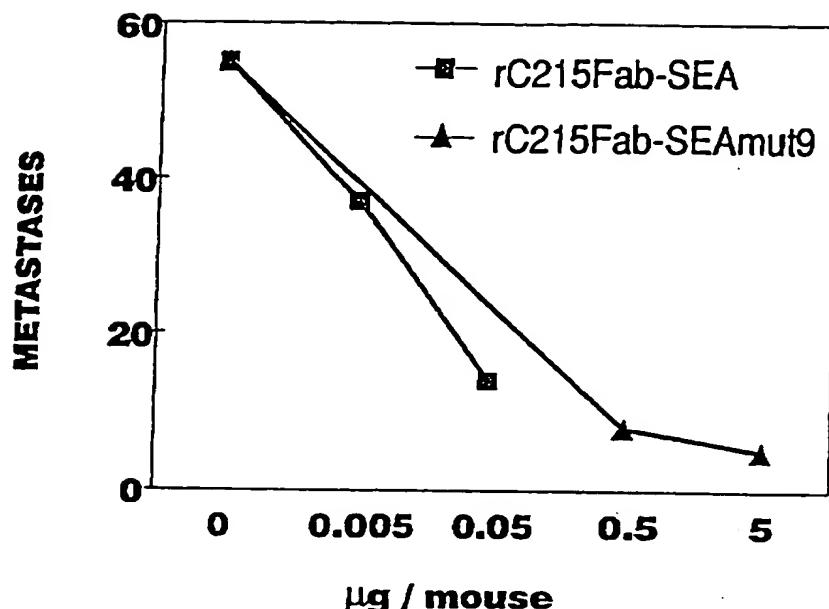


FIGURE 6A

Toxicity



FIGURE 6B

INTERNATIONAL SEARCH REPORT

International application No.

PCT/SE 95/00681

A. CLASSIFICATION OF SUBJECT MATTER

IPC6: A61K 39/00, A61K 47/48, C07K 16/46, C07K 19/00, C07K 14/31
 According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: A61K, C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

MEDLINE, EMBASE, WPI, SCISEARCH

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|-----------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------|
| X | WO 9201470 A1 (KABI PHARMACIA AB), 6 February 1992 (06.02.92), page 4, line 30 -- | 1-12 |
| X | WO 9324136 A1 (TERMAN, DAVID, S.), 9 December 1993 (09.12.93) -- | 1-12 |
| X | National Library of Medicine, File Medline, no. 92091756, (BUELOW R et al): "Localization of the immunologic activity in the superantigen Staphylococcal enterotoxin B using truncated recombinant fusion proteins", J Immunol 1992 Jan 1; 148(1): 1-6 -- | 1,2,4-8 |

Further documents are listed in the continuation of Box C.

See patent family annex.

- * Special categories of cited documents
- "A" document defining the general state of the art which is not considered to be of particular relevance
- "B" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed
- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
- "&" document member of the same patent family

Date of the actual completion of the international search

12 October 1995

Date of mailing of the international search report

23.10.95

Name and mailing address of the ISA/
 Swedish Patent Office
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INTERNATIONAL SEARCH REPORT

International application No.

PCT/SE 95/00681

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|-----------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------|
| A | <p>National Library of Medicine, File Medline, no. 94001804, (HARTWIG UF et al): "Mutations affecting MHC class II binding of the super-antigen streptococcal erythrogenic toxin A.", Int Immunol 1993 Aug; 5(8): 869-75</p> <p>--</p> | 1-12 |
| A | <p>National Library of Medicine, File Medline, no. 92043687, (GROSSMAN D et al), "Mutation of the disulfide loop in staphylococcal enterotoxin A. Consequences for T cell recognition", J Immunol 1991 Nov 15; 147(10): 3274-81</p> <p>--</p> | 1-9 |
| P,X | <p>PROC.NATL.ACAD.SCI., Volume 91, Sept 1994, Mikael Dohlsten et al, "Monoclonal antibody-superantigen fusion proteins: Tumor-specific agents for T-cell-based tumor therapy" page 8945 - page 8949</p> <p>--</p> <p>-----</p> | 1-9 |

INTERNATIONAL SEARCH REPORT

International application No.

PCT/SE95/00681

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. Claims Nos.: 13
because they relate to subject matter not required to be searched by this Authority, namely:
Claim 13 relate to a method of treatment of the human or animal body by surgery or by therapy/a diagnostic method practised on the human or animal body/Rule. 39.1.(iv).
2. Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest



The additional search fees were accompanied by the applicant's protest.



No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT
Information on patent family members

28/08/95

International application No.

PCT/SE 95/00681

| Patent document cited in search report | Publication date | Patent family member(s) | | Publication date |
|----------------------------------------|------------------|-------------------------|---------|------------------|
| WO-A1- 9201470 | 06/02/92 | AU-B- | 656906 | 23/02/95 |
| | | AU-A- | 8294191 | 18/02/92 |
| | | CA-A- | 2087164 | 21/01/92 |
| | | CN-A- | 1059278 | 11/03/92 |
| | | EP-A- | 0610179 | 17/08/94 |
| | | HU-A- | 67502 | 28/04/95 |
| | | AU-B- | 657483 | 16/03/95 |
| | | AU-A- | 8239491 | 18/02/92 |
| | | EP-A- | 0540612 | 12/05/93 |
| | | JP-T- | 6500077 | 06/01/94 |
| | | SE-A- | 9002484 | 21/01/92 |
| | | WO-A- | 9201474 | 06/02/92 |
| WO-A1- 9324136 | 09/12/93 | NONE | | |

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

REC'D 25 OCT 1996
WIPO PCT

(PCT Article 36 and Rule 70)

| | | |
|-------------------------------------------------------------------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------------------------------------|----------------------------------------------|
| Applicant's or agent's file reference Pha-1492-PCT | FOR FURTHER ACTION See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416) | |
| International application No. PCT/SE95/00681 | International filing date (day/month/year) 07.06.1995 | Priority date (day/month/year) 11.07.1994 |
| International Patent Classification (IPC) or national classification and IPC6 A 61 K 39/00, A 61 K 47/48, C 07 K 16/46, C 07 K 19/00, C 07 K 14/31 | | |
| Applicant Pharmacia AB et al | | |

| |
|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| <p>1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.</p> <p>2. This REPORT consists of a total of <u>4</u> sheets, including this cover sheet.</p> <p><input type="checkbox"/> This report is also accompanied by ANNEXES, i.e., sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).</p> <p>These annexes consist of a total of _____ sheets.</p> |
| <p>3. This report contains indications relating to the following items:</p> <ul style="list-style-type: none"> I <input checked="" type="checkbox"/> Basis of the report II <input type="checkbox"/> Priority III <input type="checkbox"/> Non-establishment of opinion with regard to novelty, inventive step and industrial applicability IV <input type="checkbox"/> Lack of unity of invention V <input checked="" type="checkbox"/> Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability, citations and explanations supporting such statement VI <input type="checkbox"/> Certain documents cited VII <input type="checkbox"/> Certain defects in the international application VIII <input type="checkbox"/> Certain observations on the international application |

| | |
|--------------------------------------------------------------------------------------------------------------------------------------------|------------------------------------------------------------------|
| Date of submission of the demand 08.01.1996 | Date of completion of this report 03.10.1996 |
| Name and mailing address of the IPEA/SE Patent- och registreringsverket Box 5055 S-102 42 STOCKHOLM Facsimile No. 08-667 72 88 | Authorized officer Jack Hedlund Telephone No. 08-782 25 00 |

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.

PCT/SE95/00681

I. Basis of the report

1. This report has been drawn on the basis of (Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments.):

the international application as originally filed.

the description, pages _____, as originally filed,
pages _____, filed with the demand,
pages _____, filed with the letter of _____,
pages _____, filed with the letter of _____

the claims, Nos. _____, as originally filed,
Nos. _____, as amended under Article 19,
Nos. _____, filed with the demand,
Nos. _____, filed with the letter of _____,
Nos. _____, filed with the letter of _____

the drawings, sheets/fig _____, as originally filed,
sheets/fig _____, filed with the demand
sheets/fig _____, filed with the letter of _____,
sheets/fig _____, filed with the letter of _____

2. The amendments have resulted in the cancellation of:

the description, pages _____

the claims, Nos. _____

the drawings, sheets/fig _____

3. This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed, as indicated in the supplemental Box (Rule 70.2(c)).

4. Additional observations, if necessary:

V. Resoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

| | | | |
|-------------------------------|--------|---------------------|-----|
| Novelty (N) | Claims | <u>6 and 8</u> | YES |
| | Claims | <u>1-5, 7, 9-12</u> | NO |
| Inventive step (IS) | Claims | | YES |
| | Claims | <u>1-12</u> | NO |
| Industrial applicability (IA) | Claims | <u>1-12</u> | YES |
| | Claims | | NO |

2. Citations and explanations

The present application refers to a conjugate comprising (a) a biospecific affinity counterpart (target-seeking group) that is capable of binding to a predetermined structure and (b) a peptide that: i) contains an amino acid sequence that is derived from a superantigen, ii) has the ability to bind to a Vb chain of a T-cell receptor and iii) has a modified ability to bind to MHC class II antigens compared to the antigen from which the peptide is derived. These parts are covalently linked together. A method for lysis of cells by using this conjugate is also included in the application.

The International Search Report revealed two documents of particular interest. Document 1, WO9201470, refers to a soluble antibody conjugate comprising an antibody linked to a structure which is recognizable by T-cells and has the ability to direct T-cells to lyse the target cell recognized by the antibody. The conjugate is characterized by the structure being a superantigen. Preferred superantigens are selected from the group of staphylococcal enterotoxins (SEs), such as SEA, SEB, SEC, SED and SEE, or active fragments and peptides thereof. Another important mode is a method for the lysis of target cells, wherein the target cells are contacted with a target cell lysis effective amount of the conjugate. The method of lysis is part of a treatment regimen for cancer, autoimmunity, parasitic infestations and fungal, viral and bacterial infections.

..../...

Supplemental Box

(To be used when the space in any of the preceding boxes is not sufficient)

Continuation of: V

Document 2, entitled "Mutations affecting MHC class II binding of the superantigen streptococcal erythrogenic toxin A" by Hartwig UF et al. Int. Immunol. Aug 1993, 5 (8) p869-75, refers to mutated Streptococcal pyrogenic exotoxin A (SPEA) which is a member of the family of superantigens produced by *Staphylococcus aureus* and *Streptococcus pyogenes*. Nine mutants were made as well as an additional variant, where 10 N-terminal amino acids had been deleted. The mutants were expressed as fusion proteins. Several mutations led to a loss of function due to the loss of class II-binding activity.

In view of document 1, obtaining a conjugate as claimed in claims 1-5, 7 and 9 is not new and therefore these claims lack novelty. The conjugate in the application as claimed in claim 1-5, 7 and 9 has not been shown to differ from the conjugate in document 1. To obtain methods for lysis of cells as claimed in claims 10-12 is also not new and these claims lack novelty as well. The conjugate as claimed in claims 6 and 8 is considered to be new and accordingly to have novelty, but it is also considered obvious for a person skilled in the art to obtain this conjugate. An inventive step can not be said to exist, especially not in view of what is said in document 2 about mutated superantigens. The conjugate claimed in claims 6 and 8 has not been shown to be non-obvious to obtain for a person skilled in the art and these claims are therefore deemed to lack an inventive step.